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STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

II. REACTIONS INVOLVING CYTOCHROME C[*]

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That a link between the coenzyme systems and cytochrome c exists in the living cell is indicated by the fact that the oxidation of the metabolites which require coenzymes is inhibited by dilute cyanide in tissue slices (2, 3). Nevertheless when Ogston and Green (4) attempted to link these metabolites with cytochrome c outside the cell, they were unsuccessful and for several years the coenzymes could be linked to oxygen only through the "old yellow enzyme" (5) which was not likely to be the natural carrier for the systems both because of its insensitivity to cyanide and its slow rate of reaction. In 1936 Theorell (6) succeeded in linking CoII to cytochrome c through the old yellow enzyme but here also the rate was much lower than that which might be expected if the mechanism were to explain the physiological reaction.

The question of the mechanism of the linkage of the coenzyme metabolites to cytochrome c was taken up at about this time by Szent-Györgyi and his coworkers, who evolved the theory of 4-carbon dicarboxylic acid catalysis (7). This theory proposes that hydrogen from the metabolites reduces fumaric acid to succinic acid, which can then be oxidized via the cytochrome system by its dehydrogenase, regenerating fumaric acid. However, experiments recently reported by Potter (8) suggest that whether or not hydrogen transport can occur via the fumarate-succinate sys-

tem, it appears that another mechanism must exist, and that the fumarate-succinate system is not an essential link in the hydrogen transport mechanism which connects CoI with cytochrome c, as had been suggested. It seems at present that the newer mechanism may include the enzyme which was discovered independently in 1937 by Adler *et al.* (9) and by Green and Dewan (10) who called the enzyme "diaphorase" and "coenzyme factor" respectively. Although both groups reported that the new enzyme was linked to the cytochromes, no proof of this concept has ever been presented, since cytochrome c reduction has not been demonstrated except with fresh crude tissue extracts. Dewan and Green (11) stated that in addition to the coenzyme factor and cytochrome oxidase, cytochromes a and b but not cytochrome c were involved in the transport of hydrogen from dihydrocozymase to oxygen. However, data to be presented below do not support that view and show that cytochrome c is needed for the reaction. The remaining problem is therefore the mechanism of the reaction between CoH_2I and cytochrome c. The experiments were supplemented with reduction tests in which succinate was the hydrogen donator.

Haas, Horecker, and Hogness (12) have recently isolated from yeast a new yellow enzyme which catalyzes the direct reaction between CoH_2II and cytochrome c, and it remains to be seen whether an analogous situation exists in the case of CoI. Preliminary experiments by Potter[1] indicate that their enzyme does not link CoI to cytochrome c, and that the CoI enzyme as prepared for the present work does not link CoII to cytochrome c.

<center>EXPERIMENTAL</center>

"Complete" Enzyme System—The method previously used for the preparation of the succinoxidase system was modified to include a more thorough elimination of substances such as hemoglobin which absorb light in wave-lengths near the cytochrome c absorption band at 550 mμ. This was accomplished by the use of alternate water and saline washing, which has been used by von Euler and Hellström (13). The method is as follows: A fresh pig heart, after the removal of fat and fascia, was passed through

[1] Unpublished data obtained at the University of Chicago in the laboratory of Professor Hogness.

a meat grinder and then through a fine Latapie mincer. The mince, which usually weighed about 100 gm., was washed alternately with water and 2 per cent NaCl (1 liter per washing) until five water and four salt washings had been completed. The time for each washing was about 5 minutes, and after each washing the mince was hand-pressed in the cloth over which it had been washed. After the final washing and pressing, the light colored residue, which weighed about 65 gm., was ground in a mortar with about 50 gm. of sand and 200 ml. of $M/15$ K_2HPO_4. The phosphate was added during a period of 20 minutes during which time all the grinding was done. The mixture was allowed to stand about an hour and was then pressed through the original cloth. The resulting extract was lightly centrifuged and the colloidal supernatant was used for the spectrophotometric experiments. All of the experiments reported were done with extracts less than 48 hours old, and the extracts were kept in the cold when not in use. The dry weights after dialysis of ten preparations fell within the range of 5 to 6 mg. per ml. The properties of the preparation were similar to those of the succinoxidase preparation previously reported (8) except that the preparation was much lower in cytochrome c and hemoglobin.

"Incomplete" Enzyme System—This enzyme was prepared from pig heart by extracting according to Stotz and Hastings (14), precipitating the extract at pH 4.6, drying the precipitate *in vacuo*, homogenizing the dry powder in alkaline phosphate, and centrifuging to obtain a colloidal supernatant. This preparation contains an active diaphorase but does not catalyze the reduction of cytochrome c by CoH_2I, as shown below. Since this work was done, the method for the isolation of Straub's flavoprotein was published (15) and this enzyme has been found by Potter[1] to constitute a more active diaphorase than the enzyme here described, while it likewise does not catalyze the reduction of cytochrome c.

Dihydrocozymase—The use of this compound obviates the necessity of using metabolites plus dehydrogenases plus coenzyme as a source of hydrogen and thus simplifies the system under consideration. The dihydrocozymase was made up by dissolving 5 mg. of cozymase in 0.5 ml. of 0.5 M Na and K phosphate buffer, pH 7.6, in a 20 ml. Thunberg tube. To this was added 5 mg. of sodium hydrosulfite in 0.5 ml. of phosphate followed by 4.0 ml. of water.

The buffer and water were previously freed from oxygen by evacuation. The mixture was then evacuated and incubated for 1 hour at 30°. The solution was then aerated for 1 or 2 minutes and the dihydrocozymase content was determined by measuring the extinction at 334 mμ before and after acidification. The extinction measurements also afforded proof that the solution was free from hydrosulfite. The solution appears to be rather unstable, and the dry preparation according to Ohlmeyer (16) has given better results. The above method was used for most of the work reported here and is convenient when only small amounts of material are available.

Cytochrome c—Dry preparations containing about 90 per cent cytochrome c (0.34 per cent Fe) were used. 0.001 M solutions of this product were made up in 0.01 N HCl and kept in the cold. The acid keeps the cytochrome completely oxidized.

Cyanide—0.01 N KCN containing 0.009 N HCl was used. This solution is alkaline enough to retain HCN but has virtually no buffering capacity.

Apparatus—The rate of cytochrome c reduction was determined by measuring the rate of change in ln I_0/I at 550 mμ by means of spectrophotometric equipment similar in principle to that described by Warburg (17) but having a tungsten filament as a light source. The light entering the reaction cell had a wave-length of 550 $\pm$ 1 mμ. The change in absorption during the reaction was measured by means of a photoelectric cell and a sensitive electrometer which was calibrated for each run by means of a rotating sector which could be adjusted to correspond to different degrees of extinction while rotating. Calibration curves were then used to convert electrometer readings into E (extinction) values, *i.e.* ln I_0/I, in terms of which all results are reported.

The measurements of the rate of dihydrocozymase oxidation were made with a similar apparatus except that the light source was a mercury arc and a quartz monochromator was used to isolate the line at 334 mμ (see Günther (18)).

Participation of Cytochrome c in Oxidation of CoH$_2$I

In the experiments reported in this section we have used only the preparation of the so called complete enzyme system. The cytochrome c content of these preparations was so low that it

could not be measured quantitatively, although all three cyto-chromes could be detected. In order to study the oxidation of CoH$_2$I, small portions of the enzyme together with buffer and all additions except dihydrocozymase were made up to a standard volume and the extinction at 334 mμ was measured. Between 60 and 90 γ of CoH$_2$I were then added, resulting in an immediate rise
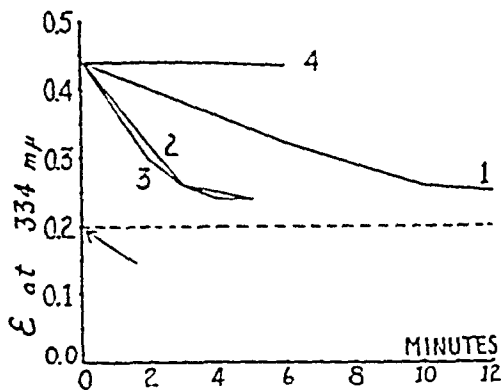
FIG. 1. Effect of added cytochrome c on the oxidation of dihydro-cozymase. Curve 1 corresponds to the following reaction mixture, 0.10 ml. of complete enzyme, 0.40 ml. of 0.5 M phosphate, pH 7.6, 3.40 ml. of water, and, after the first reading, 0.10 ml. of CoH$_2$I solution equivalent to 60 γ of CoI. The other curves correspond to the same reaction mixture as in Curve 1 except that the following additions displaced an equal amount of water, Curve 2, 0.20 ml. of 0.00002 M cytochrome c; Curve 3, 0.40 ml. of 0.00002 M cytochrome c; Curve 4, 0.20 ml. of 0.00002 M cytochrome c plus 0.40 ml. of 0.001 M neutral cyanide. All the curves are adjusted to the original extinction values indicated, which in no case is more than 0.05 unit from any of the actual observations. The arrow indicates the addi-tion of the reduced cozymase. The dotted line shows the extinction of the reaction mixtures before the reduced cozymase was added.

in extinction which then decreased with time as the dihydro-cozymase was oxidized. Various amounts of enzyme were tried and an amount which gave an oxidation time of about 10 minutes was taken as standard. The effect of additions of cytochrome c and of cyanide on the reaction was then studied. Fig. 1 shows the results of a typical experiment. It can be seen that the addi-tion of only 0.2 ml. of 0.00002 M cytochrome c (4×10^{-9} mole) produced a marked acceleration in the rate of dihydrocozymase

oxidation, and since the preparation was not completely free from cytochrome c it is believed that the slow rate of dihydrocozymase oxidation in the absence of added cytochrome is due to traces already present in the enzyme preparation.[2] Doubling the amount of added cytochrome did not appreciably increase the rate of oxidation. The fact that the reaction was blocked by the addition of cyanide in a final concentration of only 0.0001 M is a good indication that cytochrome oxidase is part of the system which brings about the oxidation of dihydrocozymase. The lowest effective concentration of cytochrome c has not been determined. From these results, however, we suggest that the normal path of hydrogen transport from dihydrocozymase to oxygen includes cytochrome c and cytochrome oxidase. Taking advantage of the fact that reactions involving cytochrome c may be studied spectrophotometrically at 550 mμ, we proceeded to study the mechanism of cytochrome c reduction, eliminating cytochrome oxidase and oxygen from the reaction chain and thereby further cutting down the number of variables in the reaction mechanism.

Reduction of Cytochrome c

Complete versus Incomplete Enzyme Systems—That diaphorase cannot react directly with cytochrome c was demonstrated by using two types of enzymes which are referred to as complete and incomplete with reference to their ability to promote the reduction of cytochrome c. In qualitative experiments it was shown that the complete system would reduce cytochrome c when CoH$_2$I was added, and the incomplete system would not, while either preparation would catalyze the anaerobic reduction of methylene blue by CoH$_2$I. After the reduction in the case of the complete system had been studied (see the next section), the earlier experiments with the incomplete system were repeated with the photo-electric apparatus. Since in the case of the incomplete enzyme the dye reduction occurred and the cytochrome c reduction did not occur (see Fig. 2), there seems to be little doubt that a factor which is present in the complete enzymes and which is not dia-

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[2] In similar experiments with complete enzyme preparations from three types of neoplasms there was no oxidation of CoH$_2$I whatsoever in the absence of added cytochrome c, while the reaction proceeded to completion when cytochrome c was added (19).

phorase (diaphorase being demonstrated on the basis of methylene blue reduction) is required for the reduction of cytochrome c by
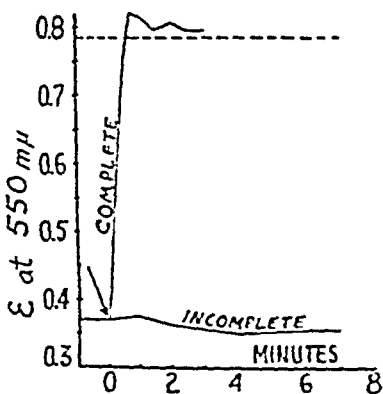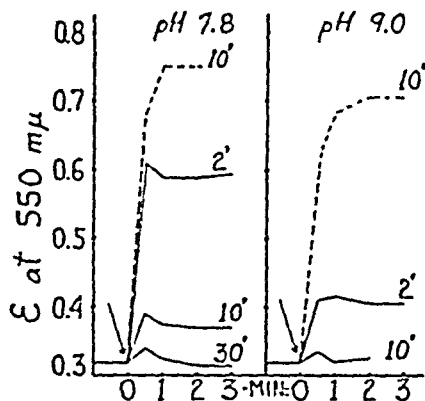
FIG. 2. The reduction of cytochrome c by dihydrocozymase in the presence of complete and incomplete enzyme preparations. In the case of the complete enzyme the cell contained the following, 0.20 ml. of 0.2 M borate, pH 7.8, 0.10 ml. of 0.001 M cytochrome c, 1.00 ml. of water, 0.30 ml. of complete Enzyme 9, 0.20 ml. of 0.01 M cyanide, and, after the first reading, 0.20 ml. of solution containing 152 γ of CoH_2I. In the case of the incomplete enzyme, 1.3 ml. of Preparation D-2 were used and no water was added. The dotted line shows the extinction of the same amount of cytochrome when reduced with $Na_2S_2O_4$. The arrow indicates the addition of reduced cozymase. The diaphorase content of the preparations was measured by their ability to promote the anaerobic reduction of methylene blue. The reaction mixtures were the same except that 0.10 ml. of 0.0005 M dye replaced the cytochrome, and the reactions were carried out at 37° instead of 20°; 0.10 ml. of complete enzyme reduced the dye in 1.5 minutes, and 1.3 ml. of incomplete enzyme reduced the dye in 3.5 minutes. In the absence of enzyme the dye was reduced in about 55 minutes.

FIG. 3. The augmentation of the strong cyanide effect by incubation and by increased pH. Each cell contained 0.20 ml. of 0.2 M borate buffer, of pH 7.8 or 9.0 as indicated above, 0.10 ml. of 0.001 M cytochrome c, 1.00 ml. of water, 0.30 ml. of complete Enzyme 7, 0.20 ml. of 0.1 M cyanide in the case of the solid lines, 0.20 ml. of 0.001 M cyanide in the case of the dotted lines, and, after the first reading, 0.20 ml. of CoH_2I solution equivalent to 130 γ of CoI in all cases as shown by the arrow. The time of incubation is indicated on the curves.

CoH_2I, while such a factor is not needed for the reduction of methylene blue. Indeed, the participation of diaphorase in the cyto-

chrome reduction remains to be shown, since its mere presence in the complete system is not enough to warrant the conclusion that it is an essential link in the transport mechanism between CoH_2I and cytochrome c.

Cyanide Inhibition of Cytochrome c Reduction—The reduction of cytochrome c was studied without the use of anaerobic conditions by adding dilute cyanide to block the action of cytochrome oxidase, which was present in the complete preparations.   It can be seen from Fig. 1 that 10^{-4} M cyanide was sufficient to block completely the oxidation in that experiment.   Experiments with succinate showed that the oxidase is effectively blocked by cyanide in final concentrations of 10^{-3} M, and that lower levels represent critical concentrations of the inhibitor.   Experiments with 10^{-2} M cyanide showed that the *reduction* of cytochrome c is inhibited by this concentration of cyanide, and it is therefore not unlikely that even 10^{-3} M cyanide has some inhibiting effect on the reduction of cytochrome c.   We have therefore used 10^{-4} M cyanide whenever this was possible in order to block the oxidase, which appears to be from 10 to 100 times as sensitive to cyanide as the cytochrome-reducing system.

Since the cyanide inhibition of cytochrome c reduction appeared to be a new observation, it was studied in somewhat greater detail, after determination of the proper amounts of CoH_2I and of succinate to be used for the reduction of 1×10^{-7} mole of cytochrome c, which was the amount best suited for the spectrophotometric measurement.   The inhibition of cytochrome c reduction was found to be dependent upon both time and pH.   The results are shown in Fig. 3, and, although omitted for the sake of brevity, perfectly analogous results were obtained in experiments in which succinate was used instead of CoH_2I.   Experiments in which the oxidase was blocked out with 10^{-4} M cyanide but in which the reduction was apparently unaffected are included for controls. It is apparent that the reduction proceeded virtually at the same rate at either pH 7.8 or 9.0, and that the inhibition by 10^{-2} M cyanide was more rapid at pH 9.0 than at pH 7.8, although the inhibition was complete even at pH 7.8 if 30 minutes incubation time were allowed before the addition of CoH_2I.   The inhibition of the oxidase by dilute cyanide on the other hand appears to be instantaneous.   It has been shown by von Euler and Hellström (13) that diaphorase is not inhibited by a pH of 9.0 in

Thunberg tests and it was shown by Adler, von Euler, and Hellström (9) that diaphorase is not inhibited by 10^{-2} M cyanide. We have confirmed both of these observations under conditions exactly parallel with our cytochrome c reduction tests. It seems clear therefore that neither the cyanide nor the pH effect involves diaphorase.

<div align="center">DISCUSSION</div>

Although the results of Ogston and Green (4) and Dewan and Green (11) do not support our conclusion that cytochrome c is an essential link in the hydrogen transport mechanism between the coenzymes and oxygen, more recent work by Hawthorne and Harrison (20) supports our position and appears to afford an explanation for the results of Dewan and Green. They demonstrated a stimulation of oxygen uptake by cytochrome c in a system involving a preparation of cytochromes a and b similar to that used by Dewan and Green by diminishing the concentration of the preparation of cytochromes a and b 20- to 40-fold. It was also shown that in the presence of large amounts of this preparation, additions of cytochrome c were without effect, as had been reported by Dewan and Green in the case of the CoI system. We feel that the results of Dewan and Green cannot be said to prove that cytochrome c is not involved in the oxidation of the coenzyme-linked metabolites until their preparation of cytochromes a and b is shown to be free from cytochrome c, and even if this is shown, one must ask whether the oxidation of CoH_2I by cytochromes a and b, if it occurs in the absence of cytochrome c, actually represents the physiological mechanism.

In attempting to analyze the mechanism by which cytochrome c is reduced, we considered that cytochrome b stood out among the various possibilities (1). A similar suggestion has also been made by Keilin and Hartree (21). In our own work, the reduction of cytochrome c took place with preparations which contained cytochrome b and failed in preparations in which cytochrome b could not be demonstrated, and in which one would expect cytochrome b to be lacking, on the basis of Keilin's work. The inhibition of cytochrome c reduction in the presence of 0.01 M cyanide also pointed to cytochrome b, since the autoxidation of cytochrome b appears to be affected by higher concentrations of cyanide (22), and since the other members of the reaction chain (diaphorase,

cytochrome c) were considered not to be affected by cyanide. Keilin and Hartree have stated that cytochrome c and cyanide do not form a complex (21, 23) although this possibility is definitely indicated in recent experiments by Potter (24).   The latter experiments furnish an adequate explanation for the cyanide effect and make unnecessary the assumption that cytochrome b is involved.

The oxidation-reduction potential of cytochrome b is well below that of cytochrome c according to Ball (25) and is well above that of CoI (26).   This fact supports the idea that cytochrome b could be part of the cytochrome c reducing mechanism, as Ball pointed out.   The potential of cytochrome b also meets the requirements of a catalyst which could link CoH_2I to fumarate anaerobically or succinate to oxygen under aerobic conditions.

The results of Dewan and Green are of further interest in connection with the theory that cytochrome b is involved in the reduction of cytochrome c by CoH_2I.   They reported that diaphorase plus CoH_2I would reduce preparations of cytochromes a and b but not cytochrome c.   Our own experience suggests that cytochrome c is reduced if the preparation of cytochromes a and b is present in addition to the diaphorase and CoH_2I.   It has not yet been possible to separate cytochromes a and b from each other or from cytochrome oxidase or indeed from diaphorase activity.   Therefore it is clearly not yet possible to conclude that the mere presence of any one of these compounds in an active preparation of what may for the moment be referred to as "cytochrome c reductase" is proof that any of the compounds are needed for the reaction, since any *one* of the constituents or some unknown constituent may be the transporting mechanism.   The indirect evidence outlined above can all be explained, however, on the assumption that CoH_2I reduces cytochrome b in the presence of diaphorase, and that the cytochrome c is then reduced by cytochrome b.   The results with succinate can also be explained on the assumption that cytochrome b is reduced by succinate in the presence of succinic dehydrogenase.   Hopkins *et al.* (27) have recently reported that succinic dehydrogenase does not react directly with cytochrome c.

The concept that the cytochromes act together to form a chain in the hydrogen transport mechanism, as suggested above, is not new, but proof of the concept has not been forthcoming, and the

work of Dewan and Green and of Ogston and Green led Martius in a recent review (28) to suggest that the various cytochromes might represent alternate paths of hydrogen transport, rather than a single path. The present work appears to refute the evidence (4, 11) upon which the Martius concept was based.

Thus far all attempts to obtain the "CoI-cytochrome c reductase" in solution have failed, and thus the preparation could not be fractionated. The isolation of the new "CoH$_2$II-cytochrome c reductase" which Haas, Horecker, and Hogness have obtained in soluble form from yeast proves that a yellow enzyme exists which is capable of reducing cytochrome c directly at physiological rates in the absence of cytochrome b. The rôle of cytochrome b in the hydrogen transport mechanism cannot at present be defined. The work of Haas, Horecker, and Hogness indicates that cytochrome b is not necessary for cytochrome c reduction, and the extreme lability of their enzyme makes it seem possible that diaphorase is a modified form of an enzyme which in its native state may possess the ability to reduce cytochrome c directly. This assumption would explain the facts as well as the assumption that cytochrome b is involved.

SUMMARY

1. The mechanism of hydrogen transport from reduced coenzyme I to oxygen was studied by means of spectrophotometric methods and evidence was obtained to show that cytochrome c is a part of the transport mechanism.

2. The mechanism of cytochrome c reduction was then studied and although indirect evidence was shown to be explainable on the assumption that CoH$_2$I reduces cytochrome b in the presence of diaphorase, with cytochrome c then reduced by cytochrome b, no definite conclusion was drawn on this point.

3. The reduction of cytochrome c was shown not to take place in the presence of diaphorase alone, and was shown to be inhibited by 0.01 M cyanide. The cyanide effect was shown to be augmented by incubation and by an increase in the pH of the medium.

fessor H. Theorell to whom we are also indebted for generous supplies of cytochrome c.   We also take this opportunity to thank Mr. G. Günther for making the spectrophotometric measurements at 334 mμ, Dr. F. Schlenk for cozymase, Dr. Erich Adler for helpful advice, and Professor H. von Euler for his continuing interest throughout this work.

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STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

III. CYANIDE INHIBITION OF CYTOCHROME C REDUCTION[*]

BY V. R. POTTER

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In 1939 Potter and Lockhart (1) reported that the enzymatic reduction of cytochrome c was blocked by 0.01 M cyanide. On the basis of this experimental observation plus the fact that diaphorase alone will not reduce cytochrome c, together with various observations reported in the literature, they suggested that cytochrome b might be necessary for the reduction of cytochrome c. Their work is reported in greater detail in Paper II (2).

Among the statements in the literature accepted at the time of these experiments was that of Keilin and Hartree (3, 4) to the effect that cytochrome c does not combine with cyanide. Since the data supporting their conclusions were not given, it may be possible that their conditions were different from those to be described below, which favor the conclusion that cytochrome c does combine with cyanide.

The present work grew out of an attempt to work out a quantitative spectrophotometric method for the estimation of cytochrome c in tumor tissue by means of the specific enzymatic reduction of cytochrome c in extracts containing other colored pigments. The plan was to use an enzyme containing succinic dehydrogenase and cytochrome oxidase. If the enzyme were allowed to act on cytochrome c in the absence of substrate, the cytochrome would be converted wholly to the oxidized form by the oxidase. The addition of a small amount of cyanide just sufficient to block the oxidase, as described in Paper II, followed by an excess of succinate, should then result in the complete conversion of the cyto-

chrome c to the reduced form.   On the basis of the change in log I_0/I at 550 mμ it was thought that the quantity of cytochrome c could be accurately determined.   When the necessary trials were made, inconsistent results appeared at first to be obtained.   It was suspected that cytochrome c was involved in a reaction with cyanide, and the experiments reported below support that conclusion.   In addition, the absorption spectrum of ferricytochrome c is shown to be altered to a slight extent by cyanide.   These experiments are not presented as an exhaustive examination of the points involved but since further investigation is beyond the scope of our research program it is felt that a brief summary of our salient findings is justified at this time.

<div align="center">EXPERIMENTAL</div>

Enzyme—The succinic dehydrogenase plus cytochrome oxidase preparation was made exactly according to the method for preparing the so called "complete" enzyme in the preceding paper. This preparation has a Q_{O_2} of zero in the absence of added substrate.

Cytochrome c—Cytochrome c was prepared from beef heart according to the method of Keilin and Hartree (3) except that it was dialyzed against distilled water instead of 1 per cent NaCl. The product was analyzed for iron according to the method of Lintzel (5) except that the colorimetry was carried out by means of the photoelectric spectrophotometer (see below) with light of wave-length 520 mμ $\pm$ 3.7 mμ.   The value for α in the expression $C = \log I_0/I/\alpha d$ under our conditions was found to be 0.850 $\times$ 10^7 cm.^{2} $\times$ gm. atoms of Fe^{-1}.   The product contained 0.184 per cent Fe and since cytochrome c contains 0.34 per cent Fe according to Keilin and Hartree (3) and Theorell (6) the product was apparently only 54 per cent pure for that form of cytochrome c which contains 0.34 per cent Fe.   The molecular extinction coefficients of the product on the basis of its iron content agreed perfectly with the values given by Theorell for reduced cytochrome at λ 520 mμ and at λ 550 mμ, and it thus follows that the preparation was likely to be free from non-cytochrome iron and pigments with absorption bands in this region of the spectrum.   The stock solution was 0.005 N with respect to HCl and was kept in the cold at all times.

Cyanide—0.10 N NaCN with HCl was prepared as in the preceding paper. Weaker solutions were prepared as needed by dilution.

Spectrophotometry—The apparatus used was a photoelectric spectrophotometer manufactured by the Central Scientific Company and referred to by them as the Cenco-Sheard spectrophotometer. Cells in which d was 1.00 cm. were used in all cases. For all the measurements on cytochrome c we used the narrowest spectral range which could be isolated practicably; namely, 5.3 mμ. This figure was obtained from the fact that the entrance slit was set at 0.7 mm. and the exit slit had a nominal width of 2.5 mμ. The main portion of the light thus had a width of 2.5 mμ, since the intensity approached zero at the extremes of the 5.3 mμ band. Values for α were determined for both ferrocytochrome c and ferricytochrome c, the former by reduction with $Na_2S_2O_4$ and the latter by oxidation with $K_3Fe(CN)_6$. Neither of the two latter compounds absorbs light in the concentrations and spectral ranges employed. When pure ferrocytochrome c or pure ferricytochrome c is being studied, it is possible to calculate the concentration (moles per ml.) simply by the following formulae in which $E = \log I_0/I$ at 550 mμ, and the subscripts o and r refer to ferri- and ferrocytochrome respectively.

$$C_r = E/\alpha_r \tag{1}$$

and

$$C_o = E/\alpha_o \tag{2}$$

However, when the cytochrome c is partly reduced and partly oxidized, it is necessary to combine these equations to obtain the fraction of the total cytochrome (C_t) present in the reduced form. Thus

$$E = C_o\alpha_o + C_r\alpha_r \tag{3}$$

and since

$$C_o + C_r = C_t \tag{4}$$

$$E = C_r\alpha_r + (C_t - C_r)\alpha_o \tag{5}$$

from which it follows that

$$C_r = \frac{E}{\alpha_r - \alpha_o} - C_t\frac{\alpha_o}{\alpha_r - \alpha_o} \tag{6}$$

Effect of Cyanide on Enzymatic Reduction of Cytochrome c

Previous experience had shown that the cyanide inhibition of cytochrome c reduction by succinate was augmented by incubation. The effect of various times of incubation with various amounts of cyanide was accordingly studied. The reaction mixture was made up to contain buffer, enzyme, cytochrome c, and cyanide and incubated in test-tubes at room temperature for various periods of time. At the end of the specified time an excess of succinate was added to reduce the cytochrome, and the mixture was transferred to an absorption cell. The values for E at λ 550 mμ were then determined and corrected for the absorption due to the enzyme. From these values the per cent of ferrocytochrome c was calculated according to Equation 6, with the extinction coefficient of "cyan-ferricytochrome c" substituted for α_o. This appears to be the correct procedure, since any ferricytochrome c present would have been reduced. The extinction of any of the mixtures encountered would accordingly be the sum of the ferrocytochrome c plus the cyan-ferricytochrome c, the extinction coefficients of which are given in the next section. The amount of enzyme was such that reduction of cytochrome was rapid for about 30 seconds, when the reaction had apparently proceeded as far as it would go since further lapse of time gave no further increase in extinction (compare with Fig. 3 in Paper II). Measurements were made until a constant value was obtained. In order to obtain values for a zero incubation time succinate was added immediately *before* the cyanide. The results of one experiment are condensed in Fig. 1.

Having found that the blocking of the cytochrome c reduction was dependent on incubation, we decided to incubate enzyme and cytochrome independently for various periods of time, adding succinate followed by enzyme or cytochrome in such a way that either the enzyme or the cytochrome was incubated with the cyanide for zero time. The results of these experiments are included in Fig. 1.

These results were repeated with two preparations of enzyme and with two preparations of cytochrome c in this laboratory. They fully confirm the results which were obtained in Stockholm and in addition provide the explanation for those results. The data indicate that cytochrome c reacts with cyanide under certain

circumstances in such a way that it cannot be reduced enzymatically. They further show that cyanide as strong as 0.01 M does not affect the enzyme systems responsible for cytochrome c reduc-
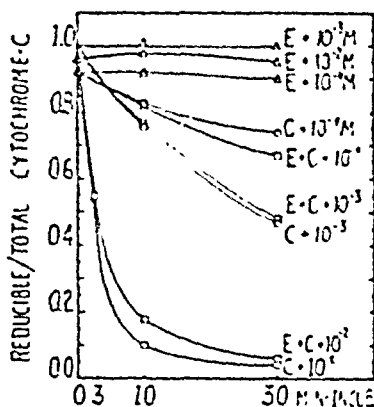
FIG. 1. The inhibition of cytochrome c reduction by cyanide. The effect of incubation of cytochrome and cyanide for various periods of time prior to the initiation of the reduction process. Each cell contained the following when log I_c/I at λ 550 mμ was determined: 1.9 ml. of water; 0.3 ml. of 0.25 M phosphate buffer, pH 7.4; 0.1 ml. of cytochrome c solution containing 0.276×10^{-7} mole of cytochrome c; 0.3 ml. of enzyme preparation, cytochrome oxidase + succinic dehydrogenase; 0.3 ml. of cyanide solution, 0.1 M, 0.01 M, or 0.001 M as indicated in the chart (10^{-3} M, etc., in the chart refers to the final molarity of the cyanide); and 0.1 ml. of 0.2 M sodium succinate. In the curves labeled E, the mixtures were made up with succinate and cytochrome omitted, and the enzyme alone was incubated with the cyanide for various periods of time. At the end of the indicated time, succinate was added, followed by the cytochrome. In the curves labeled C, the mixtures were made up with succinate and enzyme omitted, and the cytochrome alone was incubated with the cyanide. In the curves labeled $E + C$, the mixtures were made up with only succinate omitted, and both enzyme and cytochrome were incubated with the cyanide. The controls included E determinations on the following, (1) enzyme + succinate + cyanide minus cytochrome, $E = 0.103$; (2) enzyme minus succinate minus cyanide minus cytochrome, $E = 0.106$; (3) enzyme + cytochrome minus succinate minus cyanide = enzyme + ferricytochrome c, $E = 0.185$; (4) enzyme + cytochrome minus succinate minus cyanide + $Na_2S_2O_4$ = enzyme + ferrocytochrome c, $E = 0.363$; (5) enzyme + cytochrome + succinate minus cyanide, conditions anaerobic = enzyme + ferrocytochrome c, $E = 0.363$; (6) cytochrome minus enzyme, etc., + $Na_2S_2O_4$ = ferrocytochrome, $E = 0.254$. The constants used are $\alpha_r = 2.81$, $\alpha_o = 0.88$, α_o:CN = 0.96. All constants $\times 10^7$ cm.^{2} $\times$ moles^{-1}.

tion by succinate.   It seems likely that a cyan-ferricytochrome c complex is formed.   If so, this complex is formed slowly and has a large dissociation constant.   An excess of $Na_2S_2O_4$ will reduce the cyanide-cytochrome combination when succinate is ineffective, but even this reaction is slower than the reduction in the absence of cyanide.
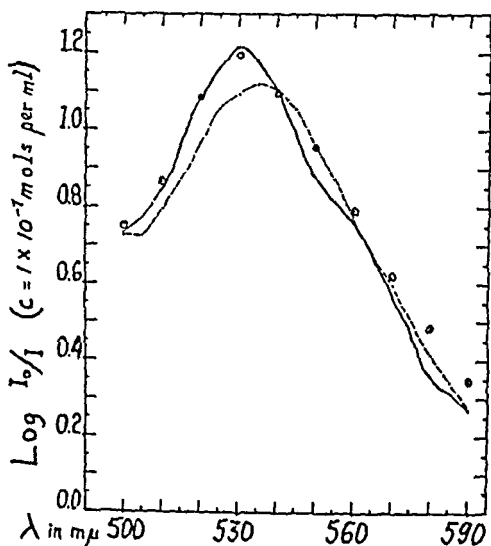
FIG. 2. Absorption spectrum of ferricytochrome c alone (solid line), and plus cyanide (dotted line).   The cyanide was incubated for 30 minutes. The concentration was calculated from the iron analysis.   Log I_0/I measured at 5 mμ intervals. $\odot$ = data from Theorell (6) for ferricytochrome c.   Measurements were made on the following mixtures, 0.3 ml. of 0.25 M phosphate buffer, pH 7.4; 0.2 ml. of cytochrome c solution containing 0.52×10^{-7} mole; 0.1 ml. of 0.01 M $K_3Fe(CN)_6$; 2.4 ml. of H_2O or 2.1 ml. of H_2O + 0.3 ml. of 0.1 N cyanide.

Absorption Spectrum of Cyan-Ferricytochrome c

Since the results in the preceding section could be explained by assuming that a cyanide-ferricytochrome complex was formed, an attempt was made to detect a change in the absorption spectrum of ferricytochrome due to cyanide.   Careful determinations of the spectrum of ferricytochrome were made, potassium ferricyanide being used to insure complete oxidation.   The concentration was determined on the basis of iron analysis and on the basis of the extinction of reduced cytochrome at 550 mμ (with

Theorell's constant), both of which gave the same result. The results check those of Theorell quite well but show lower values at 550 mμ. A possible explanation is our use of potassium ferricyanide to insure complete oxidation.

In order to convert the cytochrome to the cyanide complex similar amounts of cytochrome and ferricyanide were allowed to stand for 30 minutes in a solution which was 0.01 M with respect to cyanide, before the measurements were begun. The absorption spectra of the two compounds are shown in Fig. 2 with data on ferricytochrome taken from Theorell (6) for comparison. It is seen that in the presence of cyanide the absorption band at 530 mμ has shifted toward the red by a span of about 5 mμ. The maximum changes in absorption are about 10 per cent but these are considered to be significant since determinations of duplicate samples checked to within less than 1 per cent. These results were obtained with two other preparations of cytochrome c but Fig. 2 shows only the results of duplicate determinations of the sample for which other data have been presented. No evidence that cyanide combines with ferrocytochrome c was obtained.

DISCUSSION

The previously mentioned reports (3, 4) that ferricytochrome c does not combine with cyanide were apparently based on spectrophotometric evidence alone and the slight shift in the absorption which we have observed could be easily overlooked with many types of spectrophotometric equipment. Experiments of the type represented in Fig. 1 are, however, much more clear cut and in fact the experiments may be duplicated qualitatively in test-tubes with no spectrophotometric equipment of any kind. It would be of interest to test by this technique the other compounds which have been classified with cyanide as not combining with cytochrome c; namely, sulfide, fluoride, azide, hydroxylamine, and peroxides (3).

The results in Fig. 1 suggest that it should be possible to determine cytochrome c spectrophotometrically by means of the enzymatic oxidation and subsequent reduction of the compound, since 100 per cent recovery of the added cytochrome was obtained when reduction was carried out in the presence of 0.001 M cyanide added after the succinate.

The finding that 0.01 M cyanide blocks the reduction of cyto-chrome c by a mechanism which does not involve the reducing enzymes removes one of the lines of evidence which led to the conclusion (1) that cytochrome b was necessary for cytochrome c reduction.

SUMMARY

1. The cyanide inhibition of the enzymatic reduction of cyto-chrome c was investigated by a spectrophotometric technique. Data were presented which indicate that the enzyme is unaffected by the cyanide and that the cytochrome c is the locus of the cyanide action.

2. The absorption spectrum of ferricytochrome c was shown to be shifted toward the red by a span of 5 mμ by incubation with cyanide.

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ON THE NATURE OF THE SACCHAROID FRACTION OF HUMAN BLOOD

II. IDENTIFICATION OF GLUCURONIC ACID

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For a number of years it has been recognized that human blood contains appreciable quantities of non-fermentable copper-reducing substances which have not been completely identified. In a previous publication (1) one of us has shown that the glutathione content of tungstic acid blood filtrates can account for only approximately 37 per cent of the non-glucose reducing fraction present in such filtrates, as determined by the method of Folin and Wu. The development of a method for ridding the filtrates of glutathione, as described therein, provided an opportunity for further study of the reducing substance which remained after complete removal of glucose and glutathione from such filtrates. The term "residual saccharoid" will be used here to designate this residual reducing fraction. Attempts to precipitate this fraction with heavy metal salts were unsuccessful. Since filtrates containing this material suffered no perceptible loss of reducing power when heated to dryness on a water bath, extraction of the dry filtrate salts with appropriate solvents was employed to effect a separation of the reducing material from the complex salt mass.

EXPERIMENTAL

Tungstic acid filtrates were prepared from several liters of fresh citrated human blood which had been collected with aseptic precautions. The filtrates were treated with washed yeast and mercuric chloride to remove glucose and glutathione respectively (1), the excess mercuric salt was removed by means of hydrogen sulfide, and the resulting clear liquid evaporated to dryness on the

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water bath. Extraction experiments on the dry salts showed that the residual saccharoid could be removed quantitatively by absolute methyl alcohol, 95 per cent ethyl alcohol, and acetone, with continuous extraction for 7 to 8 hours in a Soxhlet apparatus. Ether, light petroleum, and chloroform failed to remove any of the reducing material under similar conditions. The methyl alcohol extracted the material without loss of reducing power; ethyl alcohol and acetone, however, although very effective in removing the reducing material from the salts, appeared to react with this substance during the extraction in such a way as to diminish its reducing power to 10 to 20 per cent of the expected value. The significance of this finding will be discussed below.

Extraction with absolute methyl alcohol was therefore used to concentrate the residual saccharoid and free it from the bulk of the filtrate salts. Lowering the temperature and simultaneous saturation with ether further diminished the salt content of the extracts. The organic solvents were then removed by evaporation, the residue redissolved in water, and extraction with ether or chloroform carried out to remove the contaminating fatty material. All these procedures were without effect on the reducing power of the extracts and resulted in relatively pure water-clear aqueous solutions of residual saccharoid containing from 1 to 3 mg. per cc. of reducing material expressed as glucose.

Investigation of the concentrated extracts revealed that the reducing power was completely destroyed by temperatures above 200° and by boiling with dilute alkalies. When treated with strong hydrochloric acid, furfural was evolved and detected by the aniline acetate test. After such treatment the neutralized extracts no longer reduced the Folin-Wu copper reagent. This property suggested the presence of a pentose or glucuronide. The insolubility of the material in ether does not rule out the possible presence of glucuronides, as Maughan and coworkers (2) have shown that certain glucuronides are ether-insoluble. Orcinol, phloroglucinol, and naphthoresorcinol tests were run on the extracts and all were strongly positive. Although galactose also gives positive tests with some of these reagents, its presence in this instance was ruled out by repeatedly negative mucic acid tests.

A positive Tollens naphthoresorcinol test has been interpreted by many investigators of biologic fluids as fairly definite proof of

the presence of glucuronic acid or its compounds. In the course of the present study this test was made on a number of known pentoses and glucuronides. It was found that although pentose in pure solution yields a condensation product with naphthoresorcinol which is difficultly soluble in ether as opposed to the ether-soluble pigment formed by glucuronides, the addition of small amounts of ethyl alcohol as suggested by Maughan et al. renders the pigment formed by pentoses as well as glucuronides soluble in the ether layer. Thus, the method proposed by these authors for the determination of glucuronides lacks specificity to this extent. Since the tungstic acid filtrate extracts contain traces of certain higher alcohols, it was deemed necessary to study the behavior of the Tollens naphthoresorcinol test toward pentose added to such filtrates. The standard Tollens procedure was applied simultaneously to two equal portions of concentrated extract to one of which had been added 2 mg. of arabinose, an amount roughly equivalent to the amount of reducing substance in each sample. The color which developed in the ether layer of the tube to which pentose had been added was exactly double that of the control. It must be concluded from this experiment that the filtrate extracts contain interfering substances, probably of an alcoholic nature, which render the supposedly insoluble condensation product of pentose and naphthoresorcinol soluble in ether, and thus limit the usefulness of this procedure as a differentiating test between pentoses and glucuronides under these conditions. This finding is of interest in view of the fact that Pryde and Williams (3) as well as earlier investigators mentioned by these authors have concluded that blood contains glucuronic acid on the basis of a positive Tollens naphthoresorcinol test applied to tungstic acid blood filtrates.

To eliminate the possibility that the compounds responsible for the above positive reactions might have been introduced by the yeast or formed during the prolonged heating to which the filtrates were subjected in the course of preparation of the extracts, 500 cc. of fresh sterile human blood were deproteinized in the usual manner and the resulting filtrate was reduced to a dry state entirely by freezing and subsequent vacuum desiccation at subzero temperature in an apparatus devised by Hill and Pfeiffer (4) for the preparation of desiccated serum. By this method we obtained a snowy white crystalline salt mixture in marked contrast to the dirty

yellow salt mass which resulted from prolonged evaporation of filtrates on the water bath.   5 gm. of this powder, representing 100 cc. of blood and 10 mg. of residual saccharoid, were extracted with methyl alcohol for 7 hours in a Soxhlet apparatus, the methyl alcohol evaporated under a current of air, and the residue dissolved in 5 cc. of water.   When subjected to the orcinol, phloroglucinol, and naphthoresorcinol tests, this extract gave strongly positive reactions of the same order of magnitude as was obtained in the earlier experiments.   When the glucose was then removed by fermentation with yeast and the above tests repeated, the very close quantitative correspondence with prefermentation levels showed that the substance responsible for these reactions could not have been introduced by the yeast.   This conclusion was further substantiated by similar positive tests on concentrated filtrate extracts prepared from blood which had become glucose-free by spontaneous glycolysis.

Spectroscopic examination of the amyl alcohol and ether solutions of the colored condensation products formed in the orcinol and naphthoresorcinol condensations respectively, together with arabinose and glucuronic acid controls of approximately the same reducing power, showed the characteristic absorption bands ascribed to pentoses or glucuronides, but gave no aid in differentiating between the two classes of compounds.

From another portion of concentrated glucose-free extract an osazone was prepared which crystallized in small symmetrical rosettes.   After purification by recrystallization from acetone, the melting point was 149–150° (uncorrected).   An osazone prepared from a known solution of glucuronic acid of the same reducing power as the unknown was identical in appearance and melted at 150–151°.   Intimate mixtures of the two osazones in three different proportions gave melting points ranging from 149–151°.   An osazone prepared from an arabinose solution of the same reducing strength was quite unlike the other two in appearance, crystallizing in long discrete needles characteristic of the pentosazone.

Quantitative Observations

Methyl alcohol extraction experiments on many different dried filtrates showed repeatedly that all the residual copper-reducing

material was removed from the salts and recovered in the extracts. The substance responsible for the orcinol, phloroglucinol, and naphthoresorcinol tests was simultaneously quantitatively removed. To establish the identity of the substance responsible for these color tests with that producing copper reduction, quantitative colorimetric orcinol, naphthoresorcinol, and copper reduction tests were run simultaneously on each sample of purified extract and compared with a glucuronic acid standard of approximately the same copper-reducing power as the unknown. The ratios between known and unknown showed very close agreement in all of the tests.

When the same procedure was carried out with a pentose standard of the same glucose equivalent in place of the glucuronic acid standard, the ratios for the three tests varied widely. In other words, the residual saccharoid exhibits the same chromogenic potency toward Bial's and Tollens' reagents as an equivalent amount of glucuronic acid, whereas the same amount of pentose, in terms of copper-reducing potency, yields relatively much less color in the Bial and Tollens tests.

From 5 cc. of filtrate extract containing 5 mg. of reducing substance calculated as glucose or 7.5 mg. calculated as glucuronic acid,[1] 11.1 mg. of recrystallized osazone identical in appearance and melting point with glucuronic acid osazone were obtained, which is 77 per cent of the theoretical yield of 14.3 mg.

DISCUSSION

The above data appear to indicate that glucuronic acid or its compounds are responsible for practically all of the residual reducing power of tungstic acid blood filtrates not accounted for by glucose or glutathione. A very small part of the rest reduction is due to creatinine, uric acid, and ascorbic acid; however, the total reducing value of these compounds, based on experimental determinations of their glucose equivalents, probably does not exceed 2 or 3 mg. per cent in normal bloods. When allowance is made for these compounds and for glutathione, it would appear that

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[1] According to Quick (5), 1 molecular weight of glucose has the same reducing potency as 1.5 molecular weights of glucuronic acid toward the Folin-Wu copper reagent. The reducing equivalents vary with the copper reagent employed.

human blood normally contains from 10 to 25 mg. per cent of glucuronic acid in free or conjugated form.

The constant presence of glucuronides in human blood is not at all surprising in view of the known fact that such compounds are a constituent of normal urine. Quick (6) has shown that the organism can synthesize glucuronic acid at the rapid rate of 1 gm. per hour and has suggested that the compound may play a rôle in the intermediary metabolism of carbohydrates besides acting as a detoxifying agent. The recent discovery (7, 8) that certain sex hormones are excreted as glucuronides further suggests that glucuronic acid may play a more vital rôle in body metabolism than has hitherto been supposed.

From the data at hand we are unable to draw definite conclusions concerning the form in which glucuronic acid exists in human blood. Since all of the blood glucuronide appears to reduce alkaline copper solutions even when every precaution is taken to prevent preliminary hydrolysis, it is probable that the compound exists in blood either as the free acid or as a conjugated glucuronide of the ester type.

As noted previously, 80 to 90 per cent of the residual reducing substance disappeared with regularity when filtrate salts were refluxed for an hour with ethyl alcohol or acetone. A similar loss of reducing power occurred when pure glucuronic acid solutions were subjected to the same treatment. In both cases, acid hydrolysis of the extracts effected the return of a large percentage of the reducing power, suggesting that conjugation of the glucuronic acid and the solvent occurred during the refluxing process. This finding lends support to the assumption that the compound in blood contains a free aldehyde group. Further study of the points herein involved is in progress.

SUMMARY

Glucuronic acid in free or combined form has been shown to be a constant constituent of normal human blood.

Evidence is presented that the non-glucose copper-reducing fraction of blood, as determined by the Folin-Wu method, can be almost completely accounted for by glutathione and glucuronic acid. The remaining small percentage is due to known reducing substances such as creatinine, uric acid, and ascorbic acid.

We wish to express our appreciation to Dr. J. M. Hill for his kindness in making the cold desiccation procedure available to us, and also to Dr. V. Metler for allowing us the use of the spectroscope.

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METHYLATION OF HEXOSAMINES*

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The preparation of the methylated derivatives of glucosamine and chondrosamine described in this communication was initiated because of the need of reference substances in connection with studies on the structure of mucosine and chondrosine and of other nitrogenous polysaccharides. It was anticipated that the nitrogenous moiety of the above polysaccharides might be isolated either in the form of methylated hexosamines, hexosaminic acid, or deaminized derivatives of the former.

Methylation of Chondrosamine Hydrochloride—It is generally known that methylation of glycosides proceeds more satisfactorily than that of free sugars. Unfortunately, the preparation of the chondrosamine methylglycoside proved to be a problem of its own which required considerable time and effort; therefore the direct methylation of the pentaacetate of chondrosamine was resorted to. The yield of the crude methylated product was about 2.5 to 2.75 gm. from 5 gm. of acetate, or 70 per cent of the theory. Unfortunately, only half of the material was present in crystalline form, thus reducing the yield to 35 per cent and, if we accept as 90 per cent the yield of the acetyl derivative from the hexosamine, the yield is reduced to a little over 30 per cent. Meanwhile, there appeared the very important contribution to the methylation of glucosamine by Cutler, Haworth, and Peat.[1] The yield obtained by these authors was 1 to 1.5 gm. from 5 gm. of the

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[1] Cutler, W. O., Haworth, W. N., and Peat, S., *J. Chem. Soc.*, 1979 (1937).

acetate. The authors did not specify whether this yield referred to the crystalline product or the crude material. Granting their entire product was crystalline, their result on methylation of glucosamine was slightly better than our yield on the crystalline derivative of chondrosamine, but much inferior to our yield of the crude product. Inasmuch as the program of the present study included the preparation of methylated glucosaminic acid, glucosamine hydrochloride was methylated. By the procedure described in the experimental part, the yield of the crude methylated product was 3 gm. from 5 gm. of the acetate, or 85 per cent of the theory; the yield of the crystalline material was only 2 gm. from 5 gm. of the acetate, or 57 per cent of the theory; and if 90 per cent is taken as the yield of the acetate from the glucosamine hydrochloride, the yield of the crystalline product obtained by this method is above 40 per cent of the theory. Hence it is not much lower than the yield from methylglucosaminide, the yield of the tri-O-acetylmethylglucosaminide hydrobromide from bromotri-O-acetylglucosamine hydrobromide being accepted as 52 per cent of the theory, according to Irvine, McNicoll, and Hynd.[2] In these experiments the yield of the methylated product from glucosamine was higher than that of chondrosamine. The cause of the difference lies in the nature of the pentaacetates of the substances used for methylation. The glucosamine pentaacetate was practically all crystalline, and it was readily soluble in organic solvents. Chondrosamine hydrochloride on acetylation yields only a small proportion of crystalline pentaacetate. This fraction is very insoluble and cannot conveniently be used for methylation. On the other hand, the amorphous material, which alone was used for methylation, could not be expected to be of great homogeneity. This nature of the starting material may also be responsible for the great proportion of the non-crystalline fraction of the methylated chondrosamine.

Both methylated derivatives obtained by methylation of the acetylated hexosamines were mixtures of the α and β isomers. Thus the more insoluble fraction of N-acetyltri-O-methylmethylchondrosaminide had a specific rotation of $[\alpha]_D^{20} = -12°$ (in tetrachloroethane). Dextrorotatory samples were obtained from crystallization of the mother liquors. Finally, the most soluble

[2] Irvine, J. C., McNicoll, D., and Hynd, A., *J. Chem. Soc.*, **99**, 250 (1911).

fraction, not completely free from traces of mother liquor, gave on sublimation under reduced pressure a substance with $[\alpha]_D^{20} = +113.4°$ (in tetrachloroethane).

It is peculiar that N-acetyltri-O-methylmethylglucosaminide prepared by the methylation of the acetyl derivative had $[\alpha]_D^{25} = +20°$ (in chloroform), which is very close to the value of $[\alpha]_D^{25} = +19.6°$ found by Cutler, Haworth, and Peat.[1] On the other hand, our substance showed no rotation in methanol or acetone, whereas the substance of the English workers had $[\alpha]_D = -13.1°$.

Tri-O-methylhexosaminic acids were prepared by oxidation of the corresponding tri-O-methylhexosamine hydrochlorides with mercuric oxide in a manner similar to oxidation of the corresponding hexosamine hydrochlorides to hexosaminic acid.

Cyclic Structure of Chondrosamine—The principal interest in the preparation of the methylated hexosamines was the investigation into their cyclic structure. Meanwhile, the structure of the N-acetyltri-O-methylmethylglucosaminide obtained on methylation of glucosamine was established by Haworth, Lake, and Peat[2] and confirmed by Neuberger.[4] Hence there remained the task of establishing the cyclic structure of the corresponding chondrosamine derivative. The task was accomplished by oxidation of tri-O-methylchondrosamine hydrochloride with Dakin's reagent (chloramine-T). The procedure of Herbst[5] was adopted to suit this special condition. 2,3,5-Tri-O-methyllyxose was isolated without difficulty, thus showing that the N-acetyltri-O-methylmethylchondrosaminide has the pyranoside structure. Incidentally, it may be mentioned that under the conditions employed in the present study, naphthalenesulfonchloramide presents no advantages over chloramine-T for oxidation of glucosamine.

Deacetylation of N-Acetyltri-O-Methylmethylchondrosaminide—Attempts were made to deacetylate by means of aqueous barium hydroxide. A preliminary experiment with 20 mg. of substance in 7.5 per cent solution of barium hydroxide heated in a sealed tube for 48 hours at 100° resulted in complete deacetylation. However, with larger quantities of material, the procedure did not

[3] Haworth, W. N., Lake, W. H. G., and Peat, S., *J. Chem. Soc.*, 271 (1939).

[4] Neuberger, A., *Biochem. J.*, 32, 1435 (1938).

[5] Herbst, R. M., *J. Biol. Chem.*, 119, 85 (1937).

give satisfactory results, and so the method was abandoned in favor of hydrolysis with a solution of dry hydrogen chloride gas in dry methanol.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Glucosamine Derivatives*</div>

N-Acetyltri-O-Methylmethylglucosaminide—Glucosamine penta-acetate was prepared by the procedure of Lobry de Bruyn and Van Ekenstein[6] and the crude material was used for methylation by the dimethyl sulfate method.

15 gm. lots of the crude acetate were dissolved in 15 ml. of methanol. The solution was introduced into the methylation flask which contained 60 ml. of carbon tetrachloride and 150 ml. of dimethyl sulfate. 10 ml. of water were added and the mixture was emulsified by stirring before 150 ml. of 12.5 N alkali were allowed to flow into the flask. The temperature of the water bath was 40°. The alkali was then allowed to flow at the rate of 10 drops in 15 seconds for 30 minutes and then a drop a second until 150 ml. of the alkali were added. 50 ml. of dimethyl sulfate were then added in rapid flow and 50 ml. of alkali were added at the rate of 1 drop a second. The temperature of the water bath remained at approximately 40° by the heat of the reaction. At this stage the temperature was slowly raised to 55°, half an hour being allowed to pass before further addition of the reagents. At the end of the half hour (about 3 hours after the beginning of the methylation) an additional 150 ml. of dimethyl sulfate and 200 ml. of alkali were added—the dimethyl sulfate always in advance of the alkali—at such a rate that the operation lasted about 2 hours. At the end of this time the temperature of the bath was raised to 75° and the reaction was allowed to proceed another hour or two. Further treatment was as usual. The yield of the crude material was 10 gm. from which 6.0 gm. were obtained in crystalline form; m.p. 190°, sharp (observed under the polarizing microscope).

$C_{12}H_{23}NO_6$ (277.2)

Calculated.	C 51.94,	H 8.36,	N 5.05,	OCH$_3$ 44.76,	COCH$_3$ 15.40
Found.	" 52.00,	" 8.26,	" 5.08,	" 44.77,	" 15.42

[6] Lobry de Bruyn, C. A., and Van Ekenstein, W. A., *Rec. trav. chim. Pays-Bas*, **18**, 83 (1899).

The rotation of the substance was

$$[\alpha]_D^{25} = 0.0° \text{ (in } CH_3OH \text{ or acetone)}$$

$$[\alpha]_D^{25} = \frac{+0.40° \times 100}{1 \times 2.0} = +20° \text{ (in chloroform)}$$

Tri-O-Methylchondrosamine Hydrochloride—3.0 gm. of the above glycoside were dissolved in 100 ml. of 5 per cent hydrochloric acid to which 3.0 gm. of stannous chloride were added. The solution was refluxed for 10 hours over a free flame. Hydrogen sulfide was then passed through the solution and the filtrate concentrated to dryness under reduced pressure, the temperature of the water bath not exceeding 40°. It is essential to have the residue as dry as possible before crystallization. The residue was therefore taken up in benzene, which was then removed by evaporation under diminished pressure. The operation was repeated several times and the adhering traces of benzene were removed by means of ether. The residue was finally dissolved in a minimum of hot anhydrous ethanol and ether was added to the solution until, on scratching, crystallization began on the walls of the vessel. The yield was 2.0 gm. or 75 per cent of the theory.

After two recrystallizations the crystals observed under the polarizing microscope lost their birefringence at 195° and melted at 210°.

$C_9H_{21}O_5NCl$
  Calculated. C 41.92, H 7.82, N 5.43, Cl 13.76, OCH_3 36.13
  Found.   " 41.90, " 7.98, " 5.40, " 13.66, "   36.08

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+2.32° \times 100}{1 \times 4.0} = +58° \text{ (in methanol)}$$

Tri-O-Methylglucosaminic Acid—1.5 gm. of the above substance were dissolved in 25 ml. of water. 10 gm. of mercuric oxide were added and the mixture was placed on an electric plate. After 4 minutes 25 ml. of water were added and the mixture was placed on a hot water bath for 15 minutes. At this time the red color of the mercuric oxide disappeared. The partly yellow, partly gray precipitate was removed by filtration and hydrogen sulfide was passed into the solution. This filtrate was concentrated to

dryness; the traces of moisture were removed by distilling off added benzene and later ether. It was difficult to remove the remaining traces of water; thus the residue was dissolved in a minimum amount of anhydrous alcohol which on addition of ether formed a gel consisting of felt-forming needles. Again redissolved in alcohol it formed, on removal of the solvent by distillation, a dry residue which was then recrystallized from alcohol and ether. Yield 1 gm. of substance, or 72 per cent of the theory; m.p. 178–179° under the polarizing microscope.

$C_9H_{19}O_6N$. Calculated. C 45.54, H 8.07, N 5.90, OCH_3 39.24
237.15   Found.   " 45.59, " 8.14, " 5.90,   "   39.09

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.42° \times 100}{1 \times 4.0} = +10.5° \text{ (in methanol)}$$

Oxidation of Tri-O-Methylglucosamine Hydrochloride to 2,3,5-Tri-O-Methylarabinose

Experiment I—To 2.0 gm. of 3,4,6-tri-O-methylglucosamine hydrochloride (8 mM) in 3 ml. of water 8 ml. of 1.0 N sodium hydroxide and 4.412 gm. (15 mM) of chloramine-T were added in several portions and the solution was allowed to stand 1 day at room temperature and then a day in the thermostat at 37°. 8 mM of 1.0 N hydrochloric acid were then added and the mixture was allowed to stand in the refrigerator overnight. The toluene-sulfonamide was removed by filtration, the filtrate was brought to pH 7.5 by means of a solution of barium hydroxide, a few drops of acetic acid were added, and the solution was concentrated to dryness at room temperature. When taken up in water, it left a very small insoluble residue. The mother liquor was again concentrated to dryness. At this time the residue was completely soluble in water. The aqueous solution was extracted with chloroform and the extract was dried over sodium sulfate and then was distilled, after the solvent had been removed, by distillation at a bath temperature of 110° and $p = 4 \times 10^{-5}$ mm. Yield 0.50 gm.

$C_9H_{18}O_5$ (192.1). Calculated. C 49.96, H 8.39, OCH_3 48.44
                Found.   " 50.05, " 8.40,   "   48.30

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+1.85° \times 100}{0.5 \times 10} = +37° \text{ (in 50\% ethanol)}$$

Experiment II—2 gm. of 3,4,6-tri-O-methylglucosamine hydrochloride were dissolved in 15 ml. of water containing 0.6 gm. of sodium bicarbonate and 5.0 gm. of naphthalenesulfonchloramide dissolved in 200 ml. of water were added in small portions and the reaction mixture was kept in a thermostat at 37° overnight. The reaction product was then rendered slightly acid with acetic acid and chilled in an ice-alcohol mixture. It was then filtered and the filtrate brought to pH 7.5 and concentrated to dryness under reduced pressure and at room temperature. The residue was taken up in a minimum of water, again chilled, filtered, and the filtrate extracted with chloroform. The dried chloroform extract was fractionated. The weight of the fraction distilling at a bath temperature of 110° and $p = 4 \times 10^{-5}$ mm. was 0.30 mg.—not better than treatment of an equivalent of the corresponding glucosamine hydrochloride with chloramine-T.

Experiment III—1.186 gm. of 3,5,6-tri-O-methylglucosaminic acid were dissolved in 25 ml. of water containing 5 mM of alkali and 0.860 gm. (5 mM) of chloramine-T. This was allowed to stand in the thermostat at 37° overnight, then neutralized with 5 ml. of 1.0 N hydrochloric acid, chilled in an ice-alcohol mixture, and the crystalline deposit removed by filtration. On concentration of the mother liquor a second crystalline deposit formed which again was removed by filtration. The filtrate was brought up to pH 7.5 and concentrated to a small volume and extracted with ether.

The yield of the fraction distilling at a bath temperature of 110° and $p = 4 \times 10^{-5}$ mm. was 0.300 gm.

$C_8H_{16}O_5$ (192.1). Calculated. C 49.96, H 8.39, OCH$_3$ 48.44
Found. " 49.95, " 8.16, " 48.23

Chondrosamine Derivatives

Chondrosamine Pentaacetate—3.0 gm. lots of carefully dried chondrosamine hydrochloride were acetylated by the procedure employed by Lobry de Bruyn and Van Ekenstein.[6] 6.0 gm. of the starting material yielded 9 gm. of dry material. A small part

of the acetate was insoluble in ether, slightly soluble in alcohol and benzene, and more readily soluble in chloroform. It was recrystallized from hot benzene. The melting point remained constant after two recrystallizations; m.p. 235° (at rapid heating under the polarizing microscope).

$C_{16}H_{23}O_{10}N$.   Calculated.   C 49.33, H 5.96, N 3.59, $COCH_3$ 55.26
389.2   Found.   " 49.34, " 6.02, " 3.62,   "   55.20

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.20° \times 100}{2 \times 1.0} = +10° \text{ (in chloroform)}$$

The greater part of the acetate remained as an amorphous solid mass and this material served for methylation.

N-Acetyltri-O-Methylmethylchondrosaminide—The procedure of methylation was the same as employed in the case of the penta-acetate of glucosamine. It was, however, more advantageous to employ the amorphous material because of the poor solubility of the crystalline acetate. 5 to 7 gm. lots of the acetate, as a rule, gave the best results. The yield of methylated material was 2.5 to 2.75 gm. from 5.0 gm. of acetate or 70 to 75 per cent of the theory. Unfortunately, much of the material was amorphous. The yield of pure recrystallized material was not more than half of the crude product. It may be possible in the future to purify the amorphous material. The crystalline material only was used in this investigation; m.p. 223°.

$C_{12}H_{23}O_6N$ (277.2)
    Calculated.   C 51.94, H 8.36, N 5.05, OCH_3 44.76, $COCH_3$ 15.40
    Found.   " 51.98, " 8.36, " 5.07,   "   44.73,   "   15.38

The substance had the following rotation.

$$[\alpha]_D^{25} = \frac{-0.28° \times 100}{1 \times 2.23} = -12.5° \text{ (in s-tetrachloroethane)}$$

A small sample of crude material sublimed at a bath temperature of 185°, $p = 4 \times 10^{-5}$ mm. Under the microscope it began turning into long needles (instead of small needles and larger masses at about 140°). The needles continued growing until the temperature reached 182°, when they melted.

From the mother liquors on concentration a crystalline deposit was obtained which was still slightly colored and which on sublimation at $p = 4 \times 10^{-5}$ mm. and a bath temperature of 185° had the following composition.
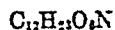
$C_{12}H_{23}O_6N$
 Calculated. C 51.94, H 8.36, N 5.05, OCH₃ 44.76, COCH₃ 15.40
 Found. " 51.98, " 8.36, " 5.07, " 44.73, " 15.38

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+3.55° \times 100}{1 \times 3.1} = +114.4° \text{ (in s-tetrachloroethane)}$$

Tri-O-Methylchondrosamine Hydrochloride—The N-acetyltri-O-methylmethylchondrosaminide was hydrolyzed by refluxing in a 5 per cent hydrochloric acid solution in the presence of stannous chloride and the reaction product worked up as in the case of the corresponding glucosamine derivative. The final substance crystallized from alcohol and ether, and to complete the crystallization pentane was added in very small portions; m.p. 178°.
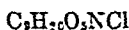
$C_9H_{20}O_5NCl$
 Calculated. C 41.92, H 7.82, N 5.43, Cl 13.76, OCH₃ 36.13
 Found. " 41.75, " 7.78, " 5.47, " 13.86, " 36.06

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+3.05° \times 100}{1 \times 2} = +152.5° \text{ (initial)} = \frac{+2.10° \times 100}{1 \times 2} =$$
$$+105° \text{ (equilibrium in } H_2O)$$

Tri-O-Methylmethylchondrosaminide—1.5 gm. of N-acetyltri-O-methylmethylchondrosaminide were dissolved in 60 ml. of methanol containing 10 per cent of dry hydrogen chloride gas and allowed to reflux on a water bath for 20 hours. The reaction product was concentrated to dryness under reduced pressure and at room temperature. The residue was taken up in anhydrous ethanol and again concentrated, the operation being repeated several times. It was finally taken up in ether and again concentrated. The final dry residue was taken up in a minimum amount of anhydrous ethanol. On addition of ether, the substance crystallized. To complete crystallization, pentane was

added dropwise. The yield of the substance was 1.368 gm. At 175° the small needles turned gradually into very long needles that melted at 227°.

$C_{10}H_{22}O_5NCl$
  Calculated. C 44.17, H 8.16, N 5.15, Cl 13.05, OCH_3 45.68
  Found. " 44.14, " 8.14, " 5.16, " 13.15, " 45.58

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+5.50° \times 100}{1 \times 3.66} = +150.3° \text{ (in methanol)}$$

Tri-O-Methylchondrosaminic Acid—0.650 gm. of tri-O-methyl-chondrosamine hydrochloride was dissolved in 25 ml. of water. 2.5 gm. of mercuric oxide were added and the suspension was heated over a free flame for 5 minutes. It was then allowed to stand on a hot water bath an additional 15 minutes. Further treatment was similar to that of the corresponding derivative of glucosamine. The yield of the substance was 0.450 gm. (75 per cent of the theory); m.p. 187° (under the polarizing microscope).

$C_9H_{19}O_6N$. Calculated. C 45.54, H 8.07, N 5.90, OCH_3 39.24
    Found. " 45.68, " 8.09, " 5.90, " 39.16

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.14° \times 100}{1 \times 2} = +7.0° \text{ (in methanol)}$$

Oxidation of Tri-O-Methylchondrosamine Hydrochloride to 2,3,5-Tri-O-Methyllyxose—2 gm. (8 mM) of tri-O-methylchondrosamine hydrochloride were dissolved in 8 ml. of 1.0 N sodium hydroxide and 4.6 gm. of chloramine-T were added in three portions. The solution was allowed to stand for 36 hours at room temperature and overnight in a thermostat at 37°. Further treatment was similar to that on oxidation of the corresponding derivatives of glu-cosamine. The yield of distilled material was 0.50 gm. The com-position showed that the material was not sufficiently pure for identification (C 49.06, H 8.11, OCH_3 45.81).

Hence the material was separated into two fractions by dis-tillation at $p = 4 \times 10^{-5}$ mm. and a bath temperature of 110°.

The composition of each of the two fractions was as follows:

$C_3H_{10}O_4$.   Calculated.   C 49.96, H 8.39, OCH_3 48.44
        Found.   Fraction I.   " 49.06, " 8.11
                  "   II.   " 50.03, " 8.26,   "   48.36, $n_D^{23} = 1.4480$

The rotation of Fraction II was

$$[\alpha]_D^{23} = \frac{-1.28° \times 100}{1 \times 7.17} = -17.8° \text{ (in 90\% ethanol)}$$

$$[\alpha]_D^{23} = \frac{+0.60° \times 100}{2 \times 0.72} = +41.7° \text{ (equilibrium in } H_2O \text{ and 8\% ethanol)}$$

ANEMIA FROM LYSINE DEFICIENCY IN DEAMINIZED CASEIN*

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It was reported in earlier papers (1, 2) that if rats receive deaminized casein as the only source of protein they survive for only a few weeks. When this protein component was reinforced with a mixture of gelatin and gliadin, the animals failed to grow, became anemic, and died. Practically the same result was obtained when either wheat gluten, corn gluten, or a laboratory preparation of lactalbumin was substituted for the gelatin-gliadin mixture. If casein, however, was included in the ration with deaminized casein, the rats grew rapidly and did not become anemic. This ration also cured animals in which anemia had already developed. Subsequently (3) it was shown that a casein hydrolysate is also effective, as is likewise the copper salt fraction of the hydrolysate which is soluble in water but insoluble in methyl alcohol. These later observations indicated that the anemia is partly due to the presence of a toxic agent in deaminized casein, and partly due to a deficiency of one or more amino acids. However, all attempts to identify the active agents by feeding pure amino acids, singly or in combination, failed. The suggestion was made that when deaminized casein is included in the ration some amino acid may be required in unusually large quantities, possibly to detoxicate the anemia-producing agent. Our more recent studies, with larger amounts of amino acids, are described in this report.

EXPERIMENTAL

If normal animals are placed on the deaminized casein rations, they do not become anemic until several weeks have elapsed,

and so the experimental periods could be greatly shortened by beginning with rats which are already anemic. This was accomplished by rearing the experimental animals on a milk ration by the method of Elvehjem and Kemmerer (4). By this procedure the effectiveness of a substance as an antianemic agent can be determined very quickly. The rations used in these studies are described in Table I.

Deaminized Casein-Wheat Gluten Ration—It was deemed necessary in earlier studies to combine deaminized casein with some

TABLE I

Composition of Rations

Constituent	Ration No.				
	3577	4033	4721*	4722*	4723*
Deaminized casein.................	10	20	20	20	
Wheat gluten......................	20				
Lysine†...........................				4	
Casein...............					20
Corn-starch..........	46.5	56.5	55.5	51.5	55.5
Milk fat.......	12.5	12.5	12.5	12.5	12.5
Salts (5)......	4.0	4.0	4.0	4.0	4.0
Agar........	2.0	2.0	2.0	2.0	2.0
Cod liver oil.....................	2.0	2.0	2.0	2.0	2.0
Water extract of yeast.............	2.0	2.0			
Tikitiki (6).......................	1.0	1.0			
Alcohol extract of dried beef liver, No. 3849 (7)....................			4.0	4.0	4.0

* In addition 200 γ of thiamine, 400 γ of riboflavin, and 300 γ of vitamin B₆ per 100 gm. of food were included.

† Calculated as the free base.

other protein that was suitable for the purpose, and wheat gluten was among the most useful of these. Ration 3577 contains this constituent. As was mentioned previously, anemia produced by deaminized casein is cured by a casein hydrolysate, and by that copper salt fraction which is soluble in water and insoluble in methyl alcohol. According to Caldwell and Rose (8) this fraction contains the essential amino acids arginine, histidine, and lysine, and in addition alanine, glutamic acid, hydroxyglutamic acid, glycine, serine, and tyrosine. It was decided to add the essential

amino acids of this group to the diet, in various combinations, and methionine was included also because an earlier trial had indicated that it might improve the rate of growth.

It will be observed in Fig. 1 that each of the combinations improved the basal diet very materially. The animals made some gains in weight, they recovered from the anemia, and though
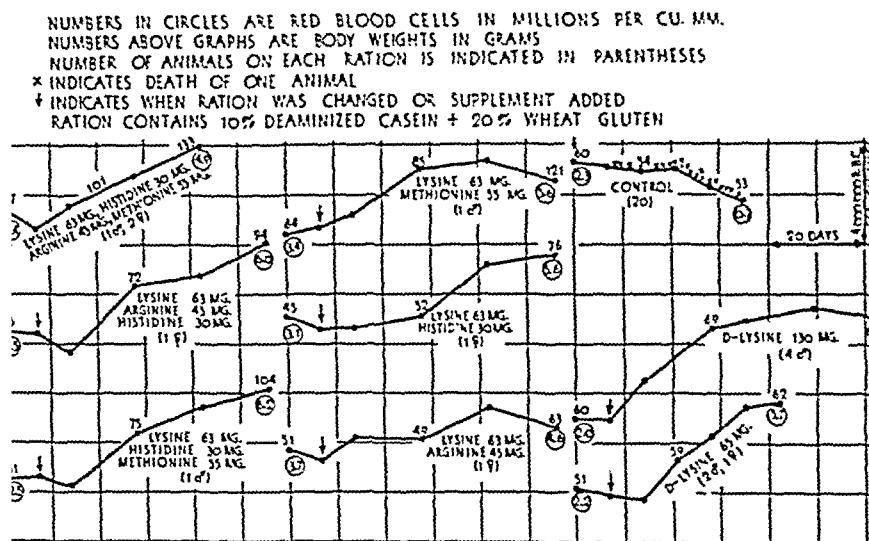
FIG. 1. The mixtures of amino acids did not cure anemia due to deaminized casein any more promptly than did lysine alone. The amino acids were offered separately from the rest of the food. The weights given in the graph are the daily allowances of the free amino acids though the hexone bases were supplied as hydrochlorides. They were mixed with enough sodium bicarbonate to neutralize the hydrochloric acid, and with a small amount of Ration 3577 to insure prompt and complete consumption. After this portion was consumed, the basal diet was supplied *ad libitum*.

there are minor variations it is apparent that all of the mixtures are about equally effective. Since the only amino acid which appears in all groups is lysine, it is concluded that it alone was responsible for the improvement. The next step was the addition of lysine alone to the basal diet. This amino acid alone is as effective as any of the combinations tried, 130 mg. daily per animal being slightly more effective than 65 mg.

Deaminized Casein As Only Protein—For the next group of experiments the ration was further simplified by omitting all protein except 20 per cent of the deaminized casein itself. Both the weights and the red blood cell counts of the control animals, on Ration 4033, declined rapidly and the animals all succumbed, as shown in Fig. 2. Apparently both declines were due to a

NUMBERS IN CIRCLES ARE RED BLOOD CELLS IN MILLIONS PER CU MM.
NUMBERS ABOVE GRAPHS ARE BODY WEIGHTS IN GRAMS
NUMBER OF ANIMALS ON EACH RATION IS INDICATED IN PARENTHESES
× INDICATES DEATH OF ONE ANIMAL
↓ INDICATES WHEN RATION WAS CHANGED OR SUPPLEMENT ADDED
RATION CONTAINS 20% DEAMINIZED CASEIN
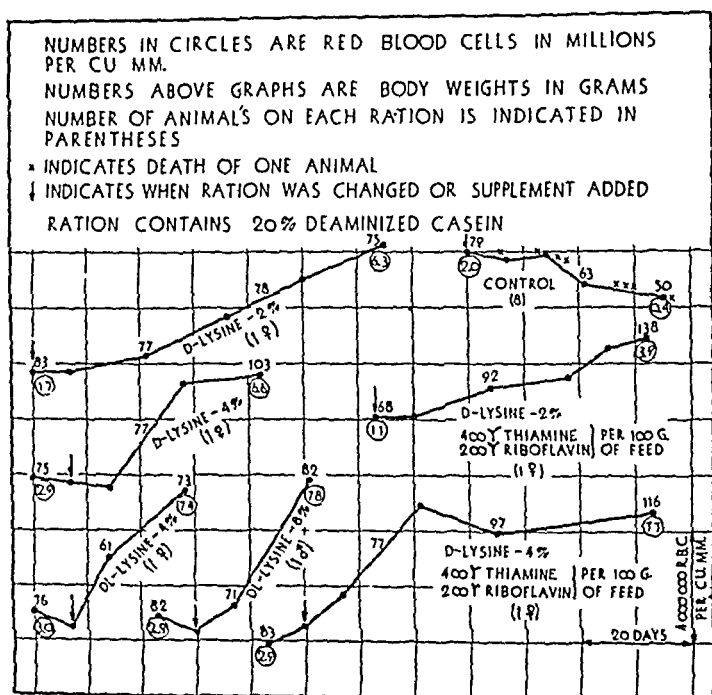
FIG. 2. The response to the control ration, No. 4033, is shown in the upper right-hand corner. The animals declined in weight and the anemia became more extreme. The rations of the other animals differed from Ration 4033 by containing lysine, which displaced an equal weight of starch. The animals which received lysine recovered from anemia. When the vitamin carriers were fortified with thiamine and riboflavin, they also made considerable gains in weight.

deficiency of lysine. When the ration contained 2 per cent of *d*-lysine, the rats recovered slowly; when it contained 4 per cent they recovered rapidly. There was a short period when *d*-lysine was not available, and *dl*-lysine was substituted for it. The rats which received 4 and 8 per cent of the racemic mixture recovered as rapidly as the rats which received 4 per cent of *d*-lysine. The

response of the one rat on 4 per cent of the mixture suggests that *l*-lysine may be effective in detoxicating deaminized casein, but additional data are required to decide that point.

It seemed surprising that rats would recover promptly from anemia and still fail to gain consistently in weight, and this observation suggested that the vitamin carriers may have deteriorated. In an attempt to improve the vitamin supply 2 per cent of milk fat was replaced by wheat germ oil and 400 γ of thiamine and 200 γ of riboflavin per 100 gm. of food were added to the ration. When the new vitamin mixture was supplied to male rats, on a diet that contained 18 per cent of casein as the only source of protein, they made an average gain of 25 gm. per week over a 6 week period. The results obtained when this vitamin mixture was incorporated in a deaminized casein-lysine ration are shown at the right of Fig. 2. Though there was marked improvement in the rate of growth, it was still subnormal.

The lysine was purchased from reliable manufacturers, but we assured ourselves that it was sufficiently pure for our purposes by determining some of the more important constants.

Comparison of Casein and Deaminized Casein plus Lysine—None of the rations that contained deaminized casein supported the optimum rate of growth, and this suggested that some essential amino acid, other than lysine, had been partly destroyed. No attempt was made to estimate the biological value of deaminized casein, but in an attempt to determine whether or not it is grossly deficient in any of the essential amino acids, it was compared, roughly, with casein.

The comparison was between Ration 4722, which contained deaminized casein and lysine, and Ration 4723, which contained casein. All rats were anemic at the beginning of the experimental period. Two of them received Ration 4722 *ad libitum*, but their food consumption was determined each day. Two others received Ration 4723, weighed out daily, but the amount was restricted to the quantity that had been consumed by the two on Ration 4722. The rates of recovery from anemia, and the gains in weight, are shown graphically in Fig. 3. The rats on the casein diet recovered from anemia more rapidly than did those that received the deaminized casein plus lysine, but the growth rates for the two groups were almost exactly the same. These data indicate that,

except in lysine, deaminized casein is not grossly deficient in any essential amino acid, and the slower growth rate is explained by a
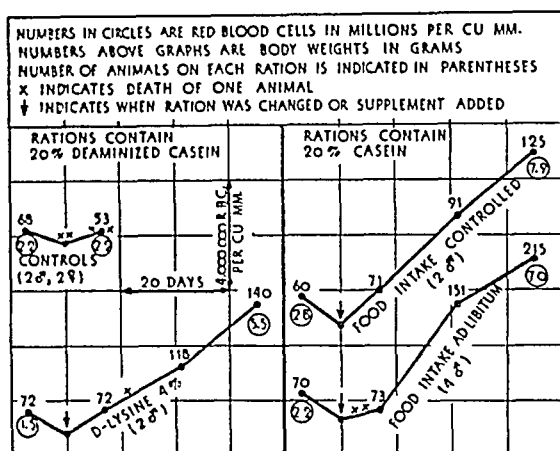
FIG. 3. The response to the control ration, No. 4721, is shown in the upper left-hand corner. The animals shown at the lower left, on Ration 4722, and at the upper right, on Ration 4723, consumed the same amount of food and made approximately the same gains in weight. The rats on the casein diet recovered normal red blood cell counts the more rapidly. It is shown at the lower right that an increased food consumption did not greatly accelerate the recovery from anemia. The data indicate that the deaminized casein-lysine mixture and casein have approximately the same biological value.

TABLE II

Gains in Weight and Food Consumption

Ration No.	No. of animals	Observations recorded	Data by weekly periods				
			1	2	3	4	5
			gm.	*gm.*	*gm.*	*gm.*	*gm.*
4721, *ad libitum*	2 M., 2 F.	Gain in weight	−13	−3			
		Food consumed	17	18			
4722 " "	2 "	Gain in weight	23	11	19	12	10
		Food consumed	41	42	43	46	46
4722, limited	2 "	Gain in weight	22	16	4	21	14
		Food consumed	41	42	43	46	46
4723, *ad libitum*	2 "	Gain in weight	14	47	32	37	27
		Food consumed	40	63	80	92	82

lowered food intake. It would be difficult to decide whether the reduced food consumption is due to the toxicity of deaminized

casein, or whether this protein is merely unpalatable. As shown in Fig. 3 four rats received Ration 4723 *ad libitum*, and their food intake was much higher than that of those which received the deaminized casein-lysine ration.

As a control, four rats were given Ration 4721, which contains deaminized casein but no lysine. They lost weight rapidly and none survived longer than 17 days. The average weekly gain in weight and food consumption of the four groups is shown in Table II.

DISCUSSION

The many earlier failures to cure anemia produced by deaminized casein with lysine must have been due to the fact that the amino acid was not given in sufficient quantity. Steudel (9) supplied only 20 mg. of lysine per rat per day. Hogan and Ritchie (1) used 25 mg. in most of their trials, although they reported one failure with 52 mg. of lysine per day. Smith and Stohlman (10) fed a mixture of tyrosine, histidine, and lysine at levels intended to be equivalent to 18 per cent of casein. At that time casein was thought to contain only about 4 per cent of lysine, so their ration must have contained less than 0.75 per cent of lysine. The results with gelatin are in accord with this. 10 per cent of gelatin, supplying 0.59 per cent lysine (11) to the ration, had failed. Our more recent results with gelatin are not described in detail, but it was observed that 20 per cent, supplying 1.18 per cent of lysine, permits recovery. According to Rose (12) the ration should contain 1 per cent of lysine in order for the rats to grow at a satisfactory rate. Our results indicate that if both growth and recovery are to occur on a deaminized casein ration over twice this much lysine must be supplied.

As to the cause of this high lysine requirement one can only speculate. Since deaminized casein presumably contains a toxic agent, a detoxication by the amino acid lysine would seem plausible. However, an attempt to isolate lysine from urine collected while the rats were being supplied with this amino acid ended in failure. Lysine could not be isolated even after strong acid hydrolysis.

Muller (13) has demonstrated a marked reticulocyte response in pigeons injected with lysine. From studies of the bone marrow

he concludes that this response is due to "stimulation and proliferation of red blood cells and an extension of blood-forming tissue." It may well be that the large amount of lysine enables the blood-forming organs to keep pace with destruction by the toxin and thus overcomes the anemia.

Hemoglobin is known to contain lysine (14); hence a lysine deficiency would prevent its synthesis. Also, the heme group itself must be synthesized, presumably from amino acids, and lysine may be a precursor.

Some comments on the earlier data concerning the antianemic activity of various proteins are suggested now that lysine is known to be the antianemic agent. Since autoclaving destroys this substance (2), it must also partly destroy lysine. The failure of many of the lactalbumin preparations (2) must have been due to thermal damage to lysine. This explanation finds added support in the observations of Greaves, Morgan, and Loveen (15) and of Waisman and Elvehjem (16). The lack of activity in corn and wheat gluten (3) is explained by their low lysine content. Gelatin failed (1) because it was supplied at too low a level.

These observations should serve to emphasize again the importance of suitable dietary protein in relation to recovery from anemia. The rôle of lysine in erythropoiesis may prove to be of importance.

SUMMARY

1. Lysine is the antianemic agent in the deaminized casein-anemia syndrome.

2. The biological method indicates that deaminized casein is not seriously deficient in any essential amino acid other than lysine.

3. If deaminized casein is the only protein in the diet and other conditions are optimal, the lysine requirement is increased two to four times the normal.

4. An interpretation of earlier data is attempted and applications of these observations are suggested.

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AN AMINO ACID CONSTITUENT OF OX BRAIN CEPHALIN

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New York)

The present paper offers evidence that from 40 to 70 per cent of the nitrogen in preparations of cephalin can be identified in an amino acid, which is probably serine. This evidence is at variance with the accepted belief that all the nitrogen is in the form of ethanolamine.

Early authors disagreed concerning the nature of the nitrogenous constituent. Thudichum (1) and Cousin (2) thought it was choline, while Koch (3) and Fraenkel and Neubauer (4) failed to find any choline in their preparations. Parnas (5), Baumann (6), and Renall (7), however, ascribed all of the cephalin nitrogen to ethanolamine, and this view has attained general acceptance (8). Only McArthur (9) claimed that an amino acid was present in the cephalin molecule. To demonstrate the presence of an amino acid among the products of acid hydrolysis of cephalin, McArthur used the method of Kober and Sugiura (10) which is based on the solubilizing action of amino acids on Cu from $Cu(OH)_2$. The method lacks specificity and no other evidence of the presence of an amino acid was offered. Probably for these reasons McArthur's work seems to have received little attention.

In the course of work on blood plasma lipids (11) the writers were brought to reconsider the nature of the nitrogenous constituent of cephalin. Material was prepared that agreed in properties and composition with the accepted criteria for cephalin. This material, without hydrolysis, was analyzed for α-amino acids by the ninhydrin-CO_2[1] method of Van Slyke and Dillon (13) as

* Fellow in Biochemistry of the Rockefeller Foundation.
[1] For a review of the reaction of ninhydrin with amino acids see Calvery (12).

modified by Van Slyke, Dillon, and MacFadyen (14). It was found in four different preparations of cephalin that from 40 to 70 per cent of the nitrogen present reacted with ninhydrin to evolve CO_2 in the manner characteristic of α-amino acids. Ethanolamine and oleic acid were found not to evolve CO_2 when treated with ninhydrin under the conditions of the determination. After acid hydrolysis of cephalin 97 per cent of the amino acid, determinable by the ninhydrin-CO_2 reaction in the intact cephalin, could be determined by the same reaction in the water-soluble products of hydrolysis.

Further evidence of the amino acid nature of the substance was shown by the fact that the reaction with ninhydrin was accompanied by liberation, with the CO_2, of an equimolar amount of NH_3, under conditions devised by MacFadyen (15) for quantitative determination of the NH_3 evolved from amino acids by this reaction. The liberation of both CO_2 and NH_3 by the reaction with ninhydrin appears to be characteristic of α-amino acids; so far as is known to the authors no other class of substances yields equimolar amounts of NH_3 and CO_2 under the conditions employed.

That the amino acid is a hydroxyamino acid was made probable by its reaction with periodate, which was introduced into amino acid chemistry by Nicolet and Shinn (16). When treated with alkaline periodate, the amino acid from cephalin yielded a molecule of ammonia, which is a reaction of amino acids that possess NH_2 and OH groups on adjacent carbon atoms (16, 17).

That the hydroxyamino acid is probably serine was shown by isolating glycolic aldehyde from the products of the reaction with ninhydrin, which has been shown without apparent exception to change the $R \cdot CH(NH_2) \cdot COOH$ of α-amino acids to $R \cdot CHO$ (18). From such a reaction only serine could yield glycolic aldehyde. The aldehyde was isolated as the dimedon compound in yield and properties identical with that obtained in control experiments from serine.

Confirmatory evidence was obtained by identifying glycolic aldehyde as a reaction product of the amino acid with chloramine-T, which Dakin (19) has shown to yield, like ninhydrin, the aldehyde with 1 less carbon atom each from glutamic acid, leucine, alanine, and glycine. The glycolic aldehyde from our product was isolated

as the phenylosazone, which was obtained in low yield, but analytically pure. No osazone was obtained from ethanolamine by the same procedure.

It appears that the hydroxyamino acid in cephalin is attached to the rest of the phosphatide molecule by ester linkage through the hydroxyl group, because reactions of the intact cephalin with nitrous acid and with ninhydrin show that the NH_2 group and the COOH group of the amino acid are both free.

That the amino acid of the cephalin is not an adsorbed impurity, but is an integral part of the lipid, may be concluded from the large amount of the amino acid found in the cephalin preparations, and from the fact that in purification the cephalin was flocculated from dilute water suspensions, in a manner which presumably separated it from water-soluble impurities.

Concerning the presence of both ethanolamine and serine in cephalin, two explanations seem possible. There may be two "cephalins" preformed in the brain; or the ethanolamine-containing cephalin may be an artifact, originating post mortem by decarboxylation of the serine constituent.

Isolation of the amino acid in pure form has not yet been accomplished; hence its identity as serine is presented as probable but not final. The results are presented at this stage because the work must be temporarily interrupted.

EXPERIMENTAL

Analytical Methods—Unless otherwise stated, manometric micromethods were used for C (20), P (21), N (22-24), and NH_2 (24, 25) estimations. Cephalin amino nitrogen was estimated by the manometric nitrous acid method as applied to lipids (24).

Preparation of Cephalin—Cephalin was prepared by the technique described below, which is a combination of the methods used by Parnas (5), Fraenkel and Neubauer (4), and Levene and Rolf (26).

10 pounds of fresh ox brains were hashed in a meat grinder and thrown into 10 liters of acetone and let stand overnight to extract non-phosphatide lipids. The next morning the acetone was filtered off and discarded. The procedure was repeated a number of times with use in succession of acetone twice (10 liters each time) and absolute ethyl alcohol once (7 liters). The solid residue was then extracted with petroleum ether twice (7 liters each time) to dissolve the cephalin. The two petroleum ether extracts were combined and concentrated in a vacuum to a volume of 800 cc. To the

concentrated extract were then added 4 liters of absolute ethyl alcohol. A precipitate of crude cephalin appeared which was collected by centrifuging, and was then redissolved in 1 liter of ethyl ether. A little water had to be added to the ether to obtain a clear solution. The ether extract was let stand overnight in the ice box. A precipitate appeared which was removed by centrifuging. The clear supernatant solution was added to 4 liters of absolute ethyl alcohol. A precipitate of crude cephalin was again formed. It was centrifuged and washed once with alcohol. The supernatant was discarded.

To remove water-soluble impurities, the crude cephalin was emulsified by rubbing with a small volume of water and then diluting gradually to 6 liters, and was then flocculated out by adding 750 cc. of N HCl. The precipitate was collected by centrifuging, washed with 0.1 N HCl, and then with acetone. The precipitate, dried at room temperature to constant weight in a vacuum, was 46 gm. It was analyzed with the following results: C 63.0 per cent, total N 1.59 per cent, amino N (with nitrous acid) 1.39 per cent, P 3.45 per cent, N:P atomic ratio 1.02, NH_2 to total N ratio 0.871.

As the NH_2:N ratio lower than 1 showed the presence of a phospholipid other than cephalin in our preparation, purification was carried further. 17 gm. were dissolved in 140 cc. of ethyl ether and the solution let stand overnight at $-6°$; the precipitate that had formed was centrifuged and the clear supernatant poured into 5 volumes of acetone. A yellowish sticky precipitate appeared which became granular on standing overnight in the ice box and then was centrifuged and washed with acetone. After being dried in a vacuum, the material weighed 8.5 gm. It had a pronounced cod liver oil odor. On analysis, the following results were obtained: C 61.4 per cent, N 1.56 per cent, P 3.90 per cent, NH_2-N 1.58 per cent, N:P atomic ratio 0.816, NH_2 to total N ratio 1.015.

6 gm. of this material were further purified. It was dissolved in 75 cc. of petroleum ether and the solution let stand for 72 hours at $-6°$. A slight precipitate formed which was centrifuged and discarded. The clear supernatant was poured into 5 volumes of acetone. 5.1 gm. of a yellowish material were obtained. A cod liver oil odor was still pronounced. This preparation was labeled A-3 and analyzed, with the following results: C 60.0 per cent, N 1.605 per cent, P 3.89 per cent, NH_2-N 1.605 per cent, N:P atomic ratio 0.916, NH_2 to total N ratio 1.00.

A second preparation, labeled C, was made from another batch of fresh brains according to the same steps used for Preparation A-3.

Two samples of calcium-cephalin were prepared according to the method of Wadsworth, Maltaner, and Maltaner (27). One, labeled Preparation Ca-A, was prepared starting from Preparation A-3; a second one, labeled Preparation Ca-B, was prepared starting from fresh brain tissue.

Reaction of Cephalin with Ninhydrin—To estimate amino acid in cephalin, samples of about 10 mg. were weighed and introduced

into tubes for carboxyl estimation. 1 cc. of water was added to each tube. From then on the ninhydrin method of Van Slyke and Dillon (13), as applied to small amounts of material by Van Slyke, Dillon, and MacFadyen (14), was followed. Blanks were run to determine CO_2 evolved (a) by boiling the cephalin with acid citrate alone, and (b) by boiling ninhydrin. The percentages of amino acid carboxyl carbon in cephalin determined by the ninhydrin-CO_2 method are multiplied by 14/12 to give values for α-amino acid nitrogen. The results are given in Table I.

Reaction of cephalin with ninhydrin was completed by immersing the tubes in boiling water for 6 minutes, the same length of time required by α-amino acids. For Preparation A-3 the results obtained, given as content of amino acid nitrogen in the

TABLE I

Presence of α-Amino Acid in Ox Brain Cephalin

Preparation	Total N	α-Amino acid nitrogen	
			Ratio to total N
	per cent	per cent	
A-3	1.605	0.965	0.601
C	1.58	0.654	0.414
Ca-A	1.60	1.092	0.682
Ca-B	1.59	0.757	0.476

cephalin, were as follows: 6 minutes, 0.965 per cent; 24 minutes, 0.962 per cent; 36 minutes, 0.966 per cent.

The amino acid content of cephalin was not affected by precipitation from aqueous solution; in the case of Preparation C, the amino acid content was estimated before and after flocculating the cephalin out of its emulsion in water, by addition of HCl. The amino acid nitrogen determined by the ninhydrin-CO_2 method before and after flocculation was respectively 0.640 and 0.665 per cent of cephalin, the difference not being significant.

Oleic acid and ethanolamine were tested to ascertain whether they formed CO_2 under the conditions of the Van Slyke-Dillon-MacFadyen (14) reaction with ninhydrin. 100 mg. of oleic acid (10 times the amount of cephalin used) did not give any increase above the blank value. Ethanolamine reacted to a significant extent only when the reaction time was increased to 4

hours, in which time it yielded 0.01 mole of CO_2. It appears that neither oleic acid nor ethanolamine interferes measurably with the determination of α-amino acids by ninhydrin under the conditions used in our analyses.

Measurement of NH_3 Accompanying CO_2 Released by Action of Ninhydrin on Cephalin

The NH_3 produced in the ninhydrin reaction with amino acids under ordinary conditions combines with the unchanged ninhydrin and the hydrindantin formed during the reaction. In order to prevent this combination and permit measurement of the NH_3, β-alanine was used according to the method of MacFadyen (15).

1 cc. of H_2O, 50 mg. of citrate buffer, 70 mg. of β-alanine, 0.8 cc. of 1 N H_2SO_4, and 35.10 mg. of intact cephalin (Preparation A-3) were placed in one of the tubes used for the ninhydrin reaction. In two similar tubes, for controls, the same reagents were placed, except that in one the ninhydrin, and, in the other, the cephalin was omitted. After evacuation at the pump the tubes were placed in the boiling water bath for the usual 6 minutes and the carboxyl carbon measured as CO_2 as usual. It was found that the ninhydrin had liberated 24.2 micromoles of CO_2 from 35.10 mg. of cephalin containing 39.9 γ atoms of N. The contents of the tubes were then washed into 50 cc. centrifuge tubes and H_2S bubbled through for 20 minutes to precipitate unchanged ninhydrin as hydrindantin. Air was passed through for 30 minutes to remove H_2S. The precipitate was removed and washed by centrifuging. Filtrate and washings were combined and made up to a volume of 15 cc. Aliquots were taken for ammonia analysis by aeration (27, 28) after 10 cc. of saturated K_2CO_3 solution were added. The ammonia was caught in 0.1 N H_2SO_4 and determined manometrically with hypobromite (22, 23).

After correction for the ammonia yielded by the blanks it was found that the ninhydrin-cephalin reaction had liberated 0.96 mole of NH_3 per mole of carboxyl CO_2. In a second experiment similarly conducted a ratio of 0.92 was obtained.

Distribution of Cephalin Nitrogen in Products of Acid Hydrolysis

242.5 mg. of cephalin Preparation A-3 were hydrolyzed by boiling under a reflux with 15 cc. of 4 N HCl for 6 hours. Fats were removed by three successive extractions with chloroform. The combined chloroform extracts were washed once with dilute HCl and the washing was added to the original extracted aqueous solution. The chloroform solution was made up to 25 cc. and labeled "chloroform-soluble fraction." The aqueous fraction was taken to dryness in a vacuum and the dry residue taken up in

25 cc. of water and labeled "water-soluble fraction." In both cases 1 cc. of solution corresponded to 9.7 mg. of parent cephalin. 5 cc. aliquots of "chloroform-soluble fraction" were taken for total N. On the "water-soluble fraction" the following determinations were performed: on 1 cc. aliquots amino acid N, on 2 cc. aliquots total N, on 4 cc. aliquots ammonia N. The samples from which ammonia had been removed by aeration were carefully acidified with HCl and transferred to 30 cc. volumetric flasks quantitatively. 5 cc. aliquots of these solutions were used for amino N estimations by the nitrous acid method (23). The results are given in Table II.

From these results it can be seen that of 0.965 gm. of amino acid N determinable by the ninhydrin-CO_2 reaction in 100 gm. of

TABLE II

Distribution of Cephalin N Following Hydrolysis by Refluxing with 4 N HCl for 6 Hours

Fraction	Nitrogen	
	As percentage of cephalin	As percentage of total cephalin N
Chloroform-soluble fraction...................	0.134	8.45
Water-soluble fraction.........................	1.445	91.10
Ammonia N..............................	0.108	6.81
Total NH_2-N (by HNO_2)....................	1.308	82.50
Amino acid HN_2-N (by ninhydrin-CO_2 reaction)...............................	0.932	58.80
Unidentified NH_2-N*.......................	0.376	23.70

* This nitrogen was probably ethanolamine, which has been identified by other observers.

parent cephalin, 0.932 gm., or 97 per cent, was determinable by the same reaction in the water-soluble fraction of the acid hydrolysate.

Identification of Aldehyde Resulting from Action of Ninhydrin on Cephalin Hydrolysate

259.7 mg. of cephalin (Preparation A-3), containing 0.965 per cent of α-amino acid N (by the ninhydrin-CO_2 method), were hydrolyzed by refluxing for 6 hours with 15 cc. of 4 N HCl. Fats were extracted with chloroform and the water-soluble fraction was taken to dryness in a vacuum. This material was then

taken up in 4 cc. of H_2O, transferred to a test-tube, and buffered to pH 2.5 with citrate buffer (50 mg.). 70 mg. of ninhydrin were added and the test-tube immersed in a boiling water bath for 6 minutes. Upon cooling, insoluble material was filtered off and to the deep purple solution were added 100 mg. of dimedon in 5 cc. of alcohol. Water was added to make a total volume of 30 cc. The solution was made slightly alkaline with dilute NaOH and then acidified with acetic acid. The test-tube was then placed in the refrigerator for 2 days at which time 24 mg. of the white crystalline dimedon were filtered off. Further standing yielded only a few more crystals.

Upon recrystallization from dilute alcohol the derivative melted at 226°. According to Vorländer (29) the dimedon compound of glycolic aldehyde melts at 227°. Carbon was estimated in this material by the wet combustion method of Van Slyke and Folch (20), C 70.80, 70.83 per cent. The theory for $C_{18}H_{24}O_4$ is 71.1 per cent carbon. From the amount of serine indicated by amino acid N determination on the cephalin used the theoretical yield of dimedon derivative of glycolic aldehyde was 54.3 mg. The actual yield of 24 mg. was therefore 45 per cent of theory.

20 mg. of *dl*-serine, when dissolved in 4 cc. of water and treated as above, yielded 28 mg. of the dimedon derivative of glycolic aldehyde, melting point 226°. The yield was 50 per cent of theory. When mixed with the dimedon compound from the amino acid of cephalin there was no depression of the melting point.

Identification of Aldehyde Resulting from Action of Chloramine-T on Amino Acid of Cephalin According to Dakin (19) after Hydrolysis Products Other Than Amino Acid Had Been Removed As Completely As Possible

A water-soluble hydrolysate from approximately 5.5 gm. of cephalin (cephalin C) was used. The hydrolysate was freed of NH_3 by aeration after being made alkaline with $Ba(OH)_2$. Ethanolamine was removed by butyl alcohol extraction of the same alkaline solution. Barium was then removed with CO_2 and glycerophosphoric acid was precipitated with lead acetate. Excess lead was removed with H_2S, and H_2S by boiling. The amino acid in the solution was then precipitated by adding mercuric acetate and sodium carbonate until no more white precipitate

appeared. Alcohol was added to 25 per cent by volume and the solution allowed to stand overnight. The precipitate was filtered off through a pad of charcoal and the mercury was removed from the precipitate by suspending in water and treating with H_2S. The filtrate was concentrated in a vacuum to 25 cc. The ninhydrin-CO_2 analysis indicated that the solution contained a concentration of α-amino acid which corresponded to 10 mg. of serine per cc.

5 cc. of this solution were made slightly alkaline with dilute NaOH and 134 mg. of chloramine-T in 5 cc. of water were added (1 mole of chloramine-T per mole of amino acid). The solution was allowed to remain at room temperature overnight and was then acidified with acetic acid. 400 mg. of phenylhydrazine hydrochloride and 600 mg. of sodium acetate in 5 cc. of water were added plus a few drops of a saturated solution of sodium bisulfite. Upon heating in the water bath for half an hour, the yellow osazone of glyoxal appeared and was filtered off. It melted at 156° but upon recrystallization several times from dilute alcohol containing sodium acetate the melting point rose to 167°. Carbon was estimated by the manometric wet combustion method (20), C 70.25, 70.26 per cent. The theory for $C_{14}H_{14}N_4$ is 70.45 per cent carbon. Glyoxal phenylosazone was prepared under identical conditions from 50 mg. of *dl*-serine and was found to melt at 167°. When mixed with the derivative from the amino acid of cephalin, there was no depression of the melting point. The same procedure was applied to 46.5 mg. of ethanolamine-chloride in 5 cc. of water. No osazone was formed.

Reaction of Amino Acid of Cephalin with Periodate

261.7 mg. of cephalin Preparation A-3 were hydrolyzed by refluxing for 6 hours with 15 cc. of 4 N HCl. Fats were removed by extraction with $CHCl_3$ and the water-soluble hydrolysate taken to dryness *in vacuo*. It was then taken up in water and made up to a volume of 25 cc. Analysis showed that this solution contained, per cc., 151 γ of total nitrogen, 87.5 γ of carboxyl nitrogen, 28.5 γ of ammonia nitrogen, and 35 γ of unidentified amino nitrogen (determined by the nitrous acid reaction (25)), which was presumably ethanolamine nitrogen. According to the method of Van Slyke, Hiller, MacFadyen, Hastings, and Klemperer

(17), a 2 cc. aliquot of the hydrolysate was placed in a large test-tube and to it were added 1 cc. of 0.2 M periodic acid, 50 mg. of glycine (to prevent the NH_3 from combining with the aldehyde simultaneously formed), and water to make a total volume of 6 cc. Then 1 cc. of 2 N NaOH was added and finally 10 cc. of saturated K_2CO_3 solution. Blanks without the hydrolysate and without the periodate were similarly prepared. The solutions were aerated for 45 minutes and the ammonia trapped in 0.1 N H_2SO_4. Ammonia was determined gasometrically (23). After correction for the blanks and preformed ammonia, it was found that periodate had liberated 219 γ of ammonia nitrogen. MacFadyen[2] has shown that under the above conditions ethanol-amine will yield 85 per cent of its nitrogen as ammonia. Assuming that the unidentified NH_2 nitrogen was in ethanolamine, it was estimated that $70 \times 0.85 = 59.5$ γ of ammonia nitrogen were formed from ethanolamine, and $219 - 59.5 = 159.5$ γ from the amino acid. Compared with the carboxyl nitrogen of the sample as determined by ninhydrin analysis, 175 γ, this calculation indicates that 91 per cent of the amino acid nitrogen was liberated as ammonia by periodate. Repetition of the experiment yielded 90 per cent of the amino acid nitrogen as ammonia.

SUMMARY

1. Cephalin prepared from ox brain has been found to contain from 40 to 70 per cent of its nitrogen as an α-amino acid. Intact cephalin reacts with ninhydrin like α-amino acids, releasing for each mole of CO_2 produced 1 mole of NH_3. After acid hydrolysis of cephalin, all the amino acid is determinable by the same reaction in the water-soluble part of the hydrolysate.

2. The amino acid has the reactions of a hydroxyamino acid. It has not been isolated, but is tentatively identified as serine by the following reactions of the water-soluble hydrolysate. (a) Treatment with ninhydrin under the conditions of the ninhydrin-CO_2 determination has yielded glycolic aldehyde, which was isolated and analyzed as the dimedon derivative, in the same yields given by control experiments with serine. (b) Treatment with chloramine-T under Dakin's conditions also yielded glycolic

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[2] MacFadyen, D. A., unpublished data.

aldehyde, which was isolated as the phenylosazone. (c) Treatment with alkaline periodate yielded ammonia. After correction for ammonia from the unidentified nitrogen fraction, the remaining ammonia was approximately 1 mole per mole of the amino acid determinable by the ninhydrin-CO_2 reaction.

3. The hydroxyamino acid is apparently attached to the cephalin molecule by an ester linkage with the hydroxyl, since both the NH_2 and the COOH groups have been found to be free in the intact cephalin.

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INTERMEDIARY METABOLISM IN DIABETES MELLITUS

ON THE SYNTHESIS OF CARBOHYDRATE FROM FAT IN THE LIVER AND FROM ACETOACETATE IN THE KIDNEY*

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The possible conversion of fatty acids into carbohydrates by mammalian tissues continues to be a controversial subject. Because carbohydrates are convertible into fats, and fats, in certain forms of plant life, are unquestionably converted into carbohydrates, the non-reversibility of this reaction in animal tissues is accepted with reluctance. Moreover, this conversion is the pivotal reaction in the overproduction hypothesis of diabetes mellitus according to which the main effect of insulin is the inhibition in the liver of the reaction, fatty acids + oxygen = carbohydrates.

Evidence concerning the possible intermediary pathways of fatty acids to carbohydrates is meager. The 2-, 4-, or 6-carbon oxidation products of fatty acids enumerated in Table I are conceivable precursors of carbohydrate. The evidence in the literature indicates that all but butyric acid, ketol, succinic acid, and acetoacetic acid can be eliminated from consideration. The first three have never been shown to be intermediaries in hepatic fatty acid catabolism. Acetoacetic acid has been shown definitely not to be a precursor of carbohydrate when perfused through the liver. Therefore since it is generally accepted that the liver is the sole site of new carbohydrate formation, the evidence for a possible pathway of fatty acid to carbohydrate is essentially nil.

TABLE I

Possible Oxidation Products of Fatty Acids in Liver and Evidence in Liver As to Their Conversion into Carbohydrate

Substance	Summary of evidence	Authority
2-Carbon compounds		
Ethyl alcohol	Not glycogenic in liver when fed	Shapiro (1935)
Acetaldehyde	Not glycogenic when fed	Sansum and Woodyatt (1915); Shapiro (1935)
Acetic acid	Not glycogenic in phlorhizinized dog	Deuel and Milhorat (1928); Ringer and Lusk (1910)
	Not glycogenic in liver except possibly with high glucose feeding	Stoehr (1933)
Ethylene glycol	Not glycogenic when fed	Shapiro (1935)
Glycol aldehyde	Possibly glycogenic; not proved	Cremer (1915); Greenwald (1918); Barrenscheen (1914); Sansum and Woodyatt (1914)
Glycolic acid	Not glycogenic when fed	Baer and Blum (1907); Greenwald (1918)
Glyoxal	" " " perfused through liver; toxic	Dakin and Dudley (1914)
Glyoxylic acid	Toxic	Adler (1906–07)
Oxalic acid	No information	
4-Carbon compounds		
Butyric acid	Not proved to be an intermediate in hepatic metabolism, but glycogenic in perfused liver	Blixenkrone-Møller (1938)
β-Hydroxybutyric acid	Not glycogenic in perfused liver	"
Acetoacetic acid	Not glycogenic in liver	"
	Glycogenic in kidney slices (?)	Weil-Malherbe (1938)
Dihydroxybutyric acid	Not glycogenic	"
Succinic acid	Glycogenic	Abundantly shown by many authors
6-Carbon compounds		
Adipic acid	Formed by ω oxidation; no information on glycogenic properties	
Ketol	Not proved to be an intermediate in hepatic metabolism, but glycogenic when fed	Stoehr and Henze (1932)

Synthesis of Carbohydrate from Acetoacetate by Kidney Slices

Recently Weil-Malherbe (1938) from experiments with rat kidney slices equilibrated *in vitro* with 0.020 M acetoacetate concluded that it was converted into glucose by this organ. He suggested that the reaction involved was an oxidative conversion of acetoacetate to pyruvate, since the latter is known to be abundantly converted into carbohydrate by kidney slices (Benoy and Elliott, 1937). This reaction would thus be a possible means of the conversion of fats to carbohydrate in mammalian tissue.

In a small series of experiments, Weil-Malherbe determined the amount of new formed *fermentable* substances, presumably carbohydrate. In five rats the mean excess of such carbohydrate formed from acetoacetate over control slices without acetoacetate was (by our calculation) 4.0 ± 2.1 micromoles of wet tissue per gm. per hour. This amount is not only small (compare 50 micromoles in the case of pyruvate (Benoy and Elliott, 1937)), but the mean value is hardly significantly different from 0. Moreover, no lactic acid determinations were reported; hence lactic acid as a possible precursor of the new carbohydrate cannot be excluded.

Our interest in the subject of fat metabolism in diabetes led us to test again the possible formation of fermentable carbohydrate from acetoacetate by kidney slices. We report in this paper a series of determinations with tissue from normal cats and rats and from diabetic cats.

EXPERIMENTAL

Diabetic Cats—The cats were depancreatized under nembutal anesthesia and were used 48 hours later. Completeness of pancreatectomy was checked by autopsy and by the degree of glycosuria.

Preparation of Kidney—The animals were killed under nembutal anesthesia and the kidneys quickly removed and dropped into cold saline. Slices of cortex of 0.4 ± 0.05 mm. thickness were prepared with a double razor blade cutter. Averaging 100 mg., they were equilibrated with 3.0 cc. of medium in 100 per cent O_2 for 2.0 hours at 38°. Each slice was divided into two parts. One-half was immediately killed and the initial value of lactic acid or fermentable carbohydrate determined. The other half was equilibrated for 2.0 hours and, together with the medium,

analyzed.   Changes in lactic acid and carbohydrates were calculated from these analyses.   In this way the effect of variations in lactic acid and carbohydrate content throughout the kidney were minimized.

Buffered Medium—The buffer used was a phosphate-saline solution of the following composition: Na_2HPO_4 0.050 M, NaCl 0.130 M, KCl 0.007 M, $MgCl_2$ 0.005 M, HCl to pH 7.2 plus substrate additions where indicated.

Determination of Total Fermentable Carbohydrate—The method used was the Shaffer-Hartmann-Somogyi, adapted for tissue carbohydrates as described by us in a previous paper (Stadie, Lukens, and Zapp, 1940).

Determination of Lactic Acid—Two methods were used.   The standard procedure was a modified Friedemann, Cotonio, and Shaffer (1927) determination, in which colloidal MnO_2 was the oxidizing agent.   We also tried the colorimetric procedure of Barker (1940) as modified by him (personal communication) and found this method to be satisfactory.   The mean recovery of lactic acid added to tissue slices was 92 ± 5 per cent by the Friedemann, Cotonio, and Shaffer method, and 99 ± 5 per cent by the colorimetric method.

The data of the experiments are presented in Table II.   Attention is called to the following points.   (1) In practically all cases, there is a slight increase of total fermentable carbohydrate.   (2) With 0.020 M acetoacetate, the increase is usually somewhat less than without acetoacetate.   In the mean the effect of acetoacetate is −2.9 ± 1.2 micromoles of fermentable carbohydrate per gm. per 2 hours.   This small difference is not significantly different from 0.   (3) In about half of the cases the *decrease* of lactic acid would account for a considerable fraction of the *increase* of carbohydrate.   This possibility was neglected by Weil-Malherbe.

We have failed to confirm Weil-Malherbe and we conclude that acetoacetic acid is not converted to carbohydrate by kidney slices from normal rats or cats or diabetic cats.

Synthesis of Carbohydrate by Liver Slices from Diabetic Cats

The evidence for the possible conversion of fatty acids to carbohydrates in the liver falls into three general categories: (1) demon-

stration in the intact normal, phlorhizinized, or diabetic animal of such low total respiratory quotients (less than 0.69) that a conversion of fatty acids to carbohydrate (R.Q. supposedly < 0.3) must be assumed; (2) demonstration of such high D:N ratios

TABLE II

Carbohydrate and Lactic Acid Metabolism of Kidney Slices from Normal and Diabetic Animals

Temperature of equilibration 38°; time 2.0 hours; phosphate-saline buffer at pH 7.2; average sample 100 mg. Supplement, 0.020 M acetoacetate where indicated.

The results are expressed in micromoles per gm. per 2 hours.

	O₂ uptake		Change from initial value					
			Lactic acid			Fermentable carbohydrate		
Animal No.	No acetoacetate	Acetoacetate	No acetoacetate	With acetoacetate	Difference	No acetoacetate	With acetoacetate	Difference
124. Normal cat	166	205	−5.6	−0.2	5.4	3.0	5.4	2.4
128A. " "			0.0	0.0	0.0	12.2	0.6	
			0.0	0.0	0.0	10.6	6.1	
Mean..............			0.0	0.0	0.0	11.4	3.3	−8.1
135A. Normal rat			−3.7	−1.8	1.9	4.3	2.3	−2.0
135B. " "			−4.2	−3.4	0.8	3.2	1.2	−2.0
120C. Diabetic cat			−7.3			3.4	1.3	
							2.0	
Mean....,...........						3.4	1.6	−1.8
122A. Diabetic cat	161	174	1.6	9.4	7.8	5.9	3.0	−2.9
122B. " "	181	165	3.1	2.2	−0.9	3.2	−2.8	−6.0
Mean effect of 0.020 M acetoacetate............			2.1 ±1.3					−2.9 ±1.2

(>3.5) in phlorhizinized or diabetic animals that the conversion must be assumed; (3) direct determination by analyses in the intact animal or perfused liver of increase of total carbohydrate in excess of possible protein, lactic acid, or glycerol precursors.

Our discussion will be limited to category (3). Chaikoff and

Weber (1928), in confirmation of the original experiments of Eppinger, Falta, and Rudinger (1908), found "beyond doubt" that the excretion of glucose in the urine of depancreatized dogs after epinephrine was far beyond that calculable from preformed carbohydrate or the protein and glycerol metabolism. Hence the extra sugar was formed from fatty acids. This experiment was repeated by Bollman, Mann, and Wilhelmj (1931) who, however, also determined the glycogen content of the muscles. They found that epinephrine causes a marked decrease in the glycogen content of the muscles of depancreatized dogs. The amount of extra glucose which appeared in the urine corresponded closely to the amount of glycogen lost from the muscles. They concluded that this was the source of extra glucose and that the conversion of fatty acids to glucose need not be assumed. The similar experiments and conclusions of Bachrach, Bradley, and Ivy (1936) and also of Chambers, Himwich, and Kennard (1935) were confirmatory of those of Bollman, Mann, and Wilhelmj.

Perfusion experiments with mammalian livers are of particular significance, since presumably the liver is the only site of formation of new carbohydrate. A complete carbohydrate balance requires the simultaneous determination of total fermentable carbohydrate, urea + ammonia, lactic acid, and ketone bodies. From such studies on twenty-two perfused cat livers, Burn and Marks (1926) concluded that there was an extra formation of carbohydrate (mean 2 to 4 mg. per gm. per hour) which could not be accounted for by known precursors except fatty acids. Gregg (1933) criticized these experiments because he found such decided irregularities in the distribution of carbohydrates in perfused livers, especially those low in carbohydrate. He was of the opinion that it is well-nigh impossible to use such a system to demonstrate glyconeogenesis from fatty acids. In seventeen perfusions of livers from fat-fed cats and dogs he found no unaccounted for new carbohydrate.

Heller (1936), using the London cannula technique in intact normal dogs fasted 3 days, measured the portal-hepatic difference of glucose and lactic acid, and the total urinary nitrogen excretion over a period of 12 hours. From the average values and an estimated hepatic blood flow, he calculated the extra carbohydrate not accounted for by protein (maximum D:N of 6.5 used) and

lactic acid. In five such experiments he found values varying from 2 to 21 micromoles per gm. per hour (mean 12 ± 4) which he concluded came from fatty acids. Although the hepatic glycogen was stated to be unchanged during the course of the experiment, the possibility that it was the precursor to the extra carbohydrate must be seriously considered in this type of experiment.

The use in this problem of liver slices equilibrated *in vitro* has been limited. Gemmill and Holmes (1935) with liver slices from fat-fed rats found a formation of new carbohydrate associated with a low respiratory quotient (mean 0.58). They concluded that these facts "both taken together, represent a strong case" for the synthesis of carbohydrates from fats. However, these low respiratory quotients, in our experience, might be completely accounted for on the basis of ketone body formation from fat. Evidence strongly opposed to the possibility of glyconeogenesis from fat is that obtained by Stadie, Zapp, and Lukens (1940) who equilibrated liver slices from diabetic cats. In such preparations they measured the oxygen uptake, the respiratory quotient, the rate of deamination of amino acids, and the ketone body formation and found that the summation of the oxygen required for all the known oxidative processes, *viz.* oxidative deamination, carbon dioxide formation, and ketone body formation, was not significantly different from the total observed oxygen uptake. In other words, the conversion of fatty acids to carbohydrates, a process which requires oxygen, could not have occurred, since there was no oxygen available in the metabolism of the slice for this conversion.

In further experiments with slices from diabetic cat livers Stadie, Lukens, and Zapp (1940) found relatively low values for new carbohydrate formation, and hence concluded that excessive carbohydrate formation did not occur in such preparations.

In the series of experiments reported here the problem was attacked more systematically. Slices of liver from diabetic cats were equilibrated and the complete carbohydrate balance was obtained by measuring the initial and final values of total fermentable carbohydrate, lactic acid, and ketone bodies. In order to correct for the formation of carbohydrate from glycogenic amino acids arising from protein catabolism, the urea and ammonia formation was also measured.

The cats were prepared as described in the preceding section of this paper.

Preparation of Liver—The animals were killed under nembutal anesthesia. The liver was quickly removed and dropped into cold saline. Slices of 0.4 ± 0.05 mm. thickness were prepared with a double razor blade cutter. Averaging 200 to 250 mg., they were placed in Warburg vessels and equilibrated in 3.0 cc. of medium and 100 per cent oxygen for 2.0 hours at 38°. The oxygen uptake and carbon dioxide output were measured by the customary Warburg technique, and are expressed as micromoles per gm. of wet tissue.

Buffered Medium—The phosphate-saline buffer had the same composition as the one described in the preceding section of this paper. The bicarbonate buffer contained $NaHCO_3$ 0.020 M, Na_2HPO_4 0.010 M, NaCl 0.110 M, KCl 0.005 M, $MgCl_2$ 0.001 M, and was equilibrated with 95 per cent O_2 and 5 per cent CO_2. The pH after equilibration was 7.1. When this buffer was used, the tissue slices were placed in 2.0 cc. of medium and were equilibrated in specially designed respirators.

Determination of total fermentable carbohydrate and of lactic acid was carried out as described in the preceding section of this paper.

Determination of Urea Plus Ammonia—The method was based on the aeration procedure and is described in a previous paper (Stadie, Lukens, and Zapp, 1940).

Determination of Ketone Bodies—The method was the slightly modified Shipley and Long (1938) procedure described in a previous paper (Stadie, Zapp, and Lukens, 1940).

Calculation of Carbohydrate Balance—The total observed increases of fermentable carbohydrate were corrected for known glycogenic products of the catabolism of the liver slices in the following ways. (1) The increase of urea + ammonia nitrogen represents the amount of amino acids which have been deaminated. On the basis of a D:N ratio of 3.0 the glucose derivable from these amino acids is as follows: glucose (moles) from glycogenic amino acids = 0.23 × increase (equivalents) of urea + ammonia nitrogen. In a large series of liver slices from diabetic cats we have found that the increase of urea + NH_3 nitrogen is quite constant and close to a mean value of 30 microequivalents per gm. of liver

per 2 hours. Accordingly we have used this value throughout. Therefore the correction = 6.9 micromoles per gm. per 2 hours. (2) Correction for change of lactic acid. When this increased, it was assumed that it came from carbohydrate. Hence the correction = $+\frac{1}{2}$ the increase in lactic acid. When lactic acid decreased, it was assumed to be converted completely to carbohydrate. Hence the correction = $-\frac{1}{2}$ the decrease in lactic acid. (3) Correction for glycerol. It was assumed that the glycerol derived from the fats *catabolized* was completely converted to carbohydrate. Ketone body formation represents total fat catabolism and on the basis of the multiple alternate oxidation hypothesis 1 mole of ketone = 0.25 mole of fatty acid. Hence the correction is glucose (moles) from glycerol = $\frac{1}{2} \times \frac{1}{4} \times \frac{1}{3} \times$ the increase (moles) of ketone bodies. The balance of the new carbohydrate after the application of these corrections would represent the carbohydrate derived from other precursors, presumably fatty acids.

The experimental data are shown in Table III. Attention is called to the following points. (1) The values of increased urea + NH_3 are close to the mean value used. (2) Lactic acid increased except when it was used as a substrate, in which case it always decreased. (3) There is no doubt of the ability of the slice to synthesize new carbohydrate from a known glycogenic precursor. With d-lactate there was an active formation of carbohydrate up to 54 micromoles per gm. per 2 hours. (4) In five out of ten experiments the amount of unaccounted for new carbohydrate ranges from -5.4 to 3.6 and the mean is -0.36 micromole per gm. per 2 hours. (5) The mean of five values of new carbohydrate fully corrected is 4.6 ± 4.3 micromoles per gm. per 2 hours. This is not significantly different from 0. The mean of all values is 5.6 ± 2.5 micromoles per gm. per 2 hours or 1.0 ± 0.5 mg. per gm. per 2 hours. This amount is hardly significantly different from 0. Whether this small amount can be attributed to fatty acid conversion is doubtful. Other possible precursors, such as preexisting glycerol or glycerophosphate, neither of which was determined, must be considered. Moreover, the amount found would correspond approximately to 0.4 gm. of glucose per kilo of body weight per day, which is to be compared with the range of glucose excretion of 3 to 5 gm. per kilo per day in de-

TABLE III

Synthesis of Fermentable Carbohydrate by Liver Slices from Diabetic Cats

Equilibration for 2.0 hours at 38°; average sample 200 to 250 mg.; buffer 3.0 cc. of phosphate-saline or bicarbonate-saline at pH 7.2. Supplements as indicated.

The results are expressed in micromoles or microequivalents per gm. per 2 hours.

Cat No.	Supplement	Oxygen (micro-moles)	n.q.	Increase of ketone bodies (micro-moles)	Increase of urea + NH_3 (micro-equivalents N)	Change in lactic acid (micromoles)	Increase of total carbo-hydrate (micromoles)	Increase of total carbo-hydrate corrected (micromoles)	Type of correction*
107CT	None	177	0.34	64	26	12.0	7.2	3.6	N, K, L
110AU	"	341*	0.32	170			15.5	1.5	" "
111EU	"	167	0.35		12	15.3	10.3	11.0	" L
111FU	"	185	0.33			17.9	9.9	11.9	" "
120BU	"	182	0.37	20	41	16.2	6.9	6.4	" K, L
120BT	"	159	0.38	20	41	3.0	1.7	−5.4	" "
111AU	0.0005 M d-lactate	165	0.45	23		−4.0	30.0	20.1	" "
111BU	0.0025 "	164	0.39	15		−18.0	15.0	−1.5	" "
111CU	0.007 "	221			30	−40.0	27.0	0.1	" L
111DU	0.007 "	209	0.53		22	−78.0	54.0	8.1	" "
Mean.........					28				

*Equilibrated 4 hrs. Summary

Samples	Type of correction	Mean corrected carbohydrate formation
5	N, K, L	4.6 ± 4.3
4	" L	7.8 ± 2.8
1	" K	1.5
10	Mean of all	5.6 ± 2.5 = 1.0 ± 0.5 mg. per gm. per 2 hrs.

pancreatized cats. These preparations contained large amounts of fatty acids (300 to 600 microequivalents per gm.). Therefore they should have abundantly produced new carbohydrates from them. From their failure to do so in half the cases and the small probably insignificant value of the mean in all the cases we conclude that the conversion of fatty acids to carbohydrate does not occur in the diabetic liver.

SUMMARY

1. The observation of Weil-Malherbe that kidney slices equilibrated *in vitro* convert acetoacetic acid to carbohydrate is not confirmed.

2. Direct measurements of the new formation *in vitro* of total fermentable carbohydrate by liver slices from diabetic cats show that the slice retains the ability to form new carbohydrate, particularly from added *d*-lactate. When the increase is corrected for possible carbohydrate formation from glycogenic amino acids, from the glycerol arising from the fat catabolized, and from changes in lactic acid, there is no significant amount of carbohydrate unaccounted for. This evidence is against the hypothesis that fatty acids are converted into carbohydrates by the diabetic liver.

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INTERMEDIARY METABOLISM IN DIABETES MELLITUS

THE NON-FORMATION OF ACETIC ACID AND THE RATIO OF KETONE BODY INCREASE TO FATTY ACID DECREASE IN LIVERS OF DIABETIC ANIMALS*

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An important problem in fat metabolism is the determination of the chemical mechanism by which the energy of fat becomes available for muscular work. There are two conceivable mechanisms: (1) *direct oxidation,* in which the oxidation of fatty acids is initiated and completed in the muscles, and (2) *indirect oxidation,* in which there is a preliminary partial oxidation by the liver of fatty acids; diffusible substrates are formed which are completely oxidized in the muscles. The possibility that both processes are operative at the same time must also be considered.

Differences of opinion arose particularly over the second hypothesis and became formulated into three alternative hypotheses: (*a*) *Successive β oxidation.* Fats are partially oxidized in the liver to ketone bodies plus a 2-carbon compound (acetic acid). Successive β oxidation may under some conditions proceed from both ends of the fatty acid chain (ω oxidation). In this case one of the end-products of the oxidation is a dicarboxylic acid. (*b*) *Multiple alternate oxidation.* Fats are partially oxidized in the liver to ketone bodies only. (*c*) *Fat to carbohydrate.* Fats are partially oxidized by the liver to carbohydrates as well as ketones. These theories have recently been discussed in detail by Stadie (1940).

The hypothesis of *β oxidation* of fatty acids was originally founded upon the work of Knoop (1904) and Dakin (1909), who

studied the excretory products after feeding phenyl-substituted fatty acids, and Embden and Marx (1907) who studied the metabolism of fatty acids when perfused through isolated livers. Dakin concluded that this was a general biochemical reaction for the oxidation of fatty acids and proposed the hypothesis of *successive β oxidation* which until recently was universally accepted. According to this the catabolism of a fatty acid group, CH_3-$(CH_2)_n$·$COOH$, is effected in the liver by the successive removal of two carbon groups at a time, with the formation of an oxidized 2-carbon compound and an intermediate fatty acid with 2 carbon atoms less than its immediate precursor. In the normal animal, this process continues until the fatty acid is completely degraded. In the diabetic, however, the oxidation stops at the 4-carbon stage, leaving a residue of 1 molecule of β-hydroxybutyric or acetoacetic acid per molecule of original fatty acid. The 2-carbon compound was usually asserted to be acetic acid. Hence the reaction for the oxidation of a typical fatty acid (palmitic) was written,

Liver of normal animal, $C_{16}H_{32}O_2 + 7O_2 = 8CH_3\cdot COOH$ (1)

Liver of diabetic animal, $C_{16}H_{32}O_2 + 6.5O_2 = C_4H_8O_3 + 6CH_3\cdot COOH$ (2)
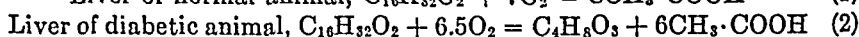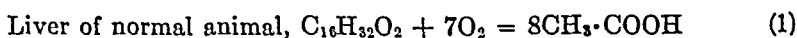
The idea that, by partial oxidation of fatty acids in the liver, acetic acid is formed which subsequently is oxidized in the muscles became firmly fixed in the literature and persists until today. For example, Embden and Michaud (1907) stated that although its formation in the liver from fatty acids has not yet been shown improved methods will make the demonstration successful. Peters and Van Slyke ((1931) p. 231) write the reaction for the oxidation of stearic acid with the formation of acetic acid. Toenniessen and Brinkmann (1938) state that without doubt large amounts of acetic acid are formed by the catabolism of fatty acids, as would be expected from Knoop's β oxidation hypothesis. They, however, were unable to demonstrate its formation in the perfused livers of rabbits. Oppenheimer and Stern ((1939) p. 256) discussing fatty acid oxidation state that "the most important reaction is Knoop's β oxidation whereby an acetic acid residue is split off from the fatty acid molecule." Mitchell ((1933) p. 484) discussed the "probable metabolites of the higher fatty acids, such as butyric and acetic acids," etc.

In an examination of the literature, however, we have been

unable to find any conclusive evidence that acetic acid is so formed in the livers of either normal or diabetic animals. In fact, aside from the negative inferences of Dakin, Embden, and Toenniessen and Brinkmann, quoted above, only one paper dealing with the subject has been found. Cook and Harrison (1936) reported large amounts of acetic acid in beef livers, but the use of an oxidizing agent in the course of their analyses makes them open to question.

In addition to the demonstration of acetic acid formation, further evidence bearing upon the nature of the oxidation of fatty acids in diabetic livers could also be obtained by determining the ratio of the increase of ketone bodies to the decrease of fatty acids during a period of active metabolism. A search of the literature fails to reveal such data.

We have previously discussed evidence (Stadie, Zapp, and Lukens, 1940) indicating that the hypothesis of successive β oxidation should be replaced by the hypothesis of multiple alternate oxidation. According to this the entire fatty acid chain is simultaneously oxidized in the liver along its whole length at alternate carbon atoms and is completely converted to ketone bodies. The reaction in the case of palmitic acid would be

$$C_{16}H_{32}O_2 + 5O_2 = 4C_4H_8O_2 \tag{3}$$

In brief the evidence is the following. (1) In livers of patients dying in diabetic acidosis, there is no proof of the presence of lower intermediary fatty acids, particularly butyric acid (Hurtley, 1916). (2) Ketone body formation by liver slices equilibrated *in vitro* with higher fatty acids was greater than that expected if 1 molecule of fatty acid yielded only 1 molecule of ketone (Jowett and Quastel, 1935). (3) The feeding of esters of higher fatty acids resulted in a greater excretion of ketone bodies than could be accounted for except on the basis of multiple alternate oxidation (Deuel, Hallman, Butts, and Murray, 1936). (4) The ratio of oxygen uptake to ketone formation in perfused liver from diabetic cats could only be accounted for if no oxidized product of fatty acid were formed other than ketone bodies (Blixenkrone-Møller, 1938). (5) The respiratory metabolism of liver slices from diabetic cats gave essentially an exact balance on the basis of this hypothesis (Stadie, Zapp, and Lukens, 1940).

Two types of supportive evidence, outlined above, remained to be determined, *viz.*: (1) Possible formation of acetic acid in liver. The diabetic animal is subsisting mainly upon fat. Therefore its liver should be actively forming acetic acid. The lack of such formation would be strong evidence against the hypothesis of successive β oxidation. (2) The molecular ratio in liver of increase of ketone bodies to the decrease of fatty acids. In the liver of the diabetic animal which is actively producing ketone bodies this ratio should be (*cf.* Equations 2 and 3) 1:1 for the hypothesis of successive β oxidation and 4:1 for multiple alternate oxidation. The experimental value of this ratio would be significant in the problem of the mechanism of fatty acid oxidation in the liver.

In this paper we present evidence bearing upon these two points. Liver slices from depancreatized (48 hours) cats or phlorhizinized normal rats or cats were equilibrated *in vitro* for periods of 2 hours and the initial and final concentrations of metabolites were compared.

EXPERIMENTAL

Depancreatized Cats—The cats were depancreatized under nembutal anesthesia, and were used 48 hours after the operation. Completeness of the pancreatectomy was checked by autopsy and by the degree of glycosuria. In two cases (Cats 139A and 139B) normal cats which gained weight on a standard diet were fed raw thyroid gland in addition to this diet for about 2 weeks prior to pancreatectomy. Under this regimen they lost weight and appeared to be hyperirritable. One of these cats (No. 139A) also received 4 mg. per kilo of dinitro-*o*-cresol subcutaneously 1 hour before being killed.

Phlorhizinized Rats—Normal male rats weighing about 250 gm. were injected with 60 mg. of phlorhizin in olive oil subcutaneously 48 and 24 hours before the experiment. During this time they were fasted and kept in metabolism cages. Marked ketonuria and glycosuria were observed.

Phlorhizinized Cats—The cats were fasted for 48 hours and then injected with 1 gm. of phlorhizin in olive oil subcutaneously on 2 successive days, during which time fasting was continued. They were used 24 hours after the last phlorhizin injection.

Preparation of Liver—The animals were anesthetized with nembutal and the livers were quickly removed and dropped into cold saline. Liver slices of 0.40 ± 0.05 mm. were prepared with a double razor blade cutter. Averaging 250 mg., they were placed in Warburg vessels in 3.0 cc. of medium and 100 per cent oxygen. The oxygen consumption and carbon dioxide production were measured for a 2.0 hour period at 38° by the customary Warburg technique, and are expressed as micromoles per gm. of wet tissue. The time elapsing between removal of the liver and commencement of the equilibration at 38° averaged 20 minutes.

Buffered Medium—Two phosphate-saline media were used. One buffer contained Na_2HPO_4 0.050 M, NaCl 0.130 M, KCl 0.007 M, $MgCl_2$ 0.005 M, and HCl to pH 7.2 plus substrate additions where indicated. The other buffer contained Na_2HPO_4 0.030 M, NaCl 0.090 M, KCl 0.005 M, $MgCl_2$ 0.005 M, and HCl to pH 7.2 plus substrate additions where indicated. As there were no essential differences in the metabolism of liver slices in these two buffers, they are not distinguished in Tables I and II.

Determination of Acetic Acid (Steam-Volatile Acid)—The contents of the Warburg vessels were transferred to centrifuge tubes, the liver macerated, and the protein precipitated with tungstic acid. Acetic acid was determined on these tungstic acid filtrates by steam distillation from a small glass-stoppered distilling flask. The sample (4.0 cc.) was acidified with 0.5 cc. of 15 M H_3PO_4 and steam admitted to the distilling flask. A 660 watt heating coil controlled by a rheostat was placed under the flask and the heat adjusted so as to maintain constant volume during the distillation. 25 cc. of distillate were collected, and the entire distillate, or an aliquot, was titrated with 0.01 N NaOH from a Rehberg burette with phenolphthalein as indicator. CO_2-free air was bubbled through the distillate for 3 minutes before and during the titration to minimize the CO_2 error. All glassware used in the distillation was thoroughly steamed before use.

By this method all steam-volatile acids (including the saturated aliphatic acids up to 8 carbon atoms) can be determined. Pyruvic, lactic, acetoacetic, and β-hydroxybutyric acids do not interfere in the concentrations usually encountered. The recovery of steam-volatile acids from tissue would necessitate further procedures to identify the acid or acids in the distillate. This

problem, however, did not arise in the present experiments, as essentially no steam-volatile acid was found.

On the average 88 per cent of acetic acid added to liver slice extract could be recovered by the method when the quantity of acetic acid distilled was 5 to 20 micromoles (0.30 to 1.20 mg.). As little as 1 micromole (0.06 mg.) of acetic acid in 25 cc. of distillate could be detected, although the increase in titration over the water blank was too small to give reliable quantitative recovery.

Determination of Ketones—Acetoacetic acid and β-hydroxybutyric acid were determined by the slightly modified Shipley and Long (1938) method described in a previous paper (Stadie, Zapp, and Lukens, 1940). The sum is expressed as micromoles of wet tissue per gm.

Determination of Fatty Acids—The method used was an adaptation of the method described by Stoddard and Drury (1929) for total fatty acids in blood. After the respiration period the contents of the Warburg flask were transferred to a 100 cc. flask and the liver finely macerated. The resulting suspension was neutralized and extracted three times for 15 minutes each with 20, 20, and 10 cc. of boiling alcohol-ether (3:1) mixture under reflux condensers. The alcohol-ether extracts were then saponified, and the fatty acids isolated and titrated essentially as described by Stoddard and Drury. All fatty acids of 6 or more carbon atoms are determined by this method.

The method was quite reproducible for the recovery of known amounts of fat, as is indicated by the following results of determinations on 30 mg. samples of bayberry wax: 109.2, 109.5, 110.8, 117.6, 107.6, 108.0, 113.4; mean 110.9 ± 1.4 microequivalents of fatty acid recovered.

Method of Sampling—In view of the known variation in the amount of fat in different sections of the same liver, duplication of the amount of fat in different liver slices from the same animal was not to be expected. Each slice therefore was divided in two, one part being used for equilibration in Warburg vessels, while the companion part was used for the determination of the initial amount of fatty acid in the slice. Thus each Warburg vessel had a corresponding initial amount from which the changes in liver fat were calculated.

Possible Formation of Acetic Acid in Liver

The data of this experiment are shown in Table I. The following points are emphasized. (1) There was a considerable ketone body formation. (2) There was essentially no acetic acid formation. By the method of steam distillation fatty acids up to

TABLE I

* *Oxygen Uptake, Respiratory Quotient, Ketone Body Formation, and Acetic Acid Formation of Liver Slices from Depancreatized (48 Hours) Cats*

$t = 38°$; average sample 200 to 250 mg.; 3.0 cc. of phosphate-saline buffer (pH 7.2); equilibrated 2.0 hours. Supplements as indicated.

The results are expressed in micromoles per gm. per 2 hours.

Cat No.	O_2 uptake	n.q.	Total ketone body formation	Acetic acid formation	
				Calculated*	Observed
106A	146	0.30	32		
	136	0.35			1.1
	141	0.42	25		
	146	0.33			0.6
	159†	0.47	23		
	158†	0.52			0.5
	168†	0.45	23		
	154†	0.56			1.0
Mean..........			25	150	0.8
106B	186	0.23	66		0.0
	183	0.21	84		
	184	0.23	61		0.0
	179	0.24	80		
	206†	0.26	91		0.0
	161†	0.29	67		
	202†	0.30	71		0.0
	173†	0.38	71		
Mean..........			74	444	0.0
110A‡	338	0.28	170		
	334	0.29	59§		
	354	0.29	180		
	350	0.28	60§		
	326	0.42			0.0
	351	0.38			0.0
Mean..........			175	1050	0.0

TABLE I—*Concluded*

Cats receiving insulin subcutaneously 1–2 hrs. before preparation of liver slices					
Cat No.	O₂ uptake	R.Q.	Total ketone body formation	Acetic acid formation	
				Calculated*	Observed
107A	145	0.35	30		1.9
	147	0.33	32		
	143	0.29	34		
	152	0.29	28		
	175†	0.47	20		
	173†	0.46			2.5
	165†	0.45	16		
	206†	0.41	20		1.3
Mean........................			26	154	1.9
107B	130	0.62	2		
	126	0.64	2		
	133	0.66			0.0
	124	0.67			0.0
107C	175	0.34			0.0
	173	0.37			0.0
	159	0.34	31		
	188	0.35	35		
Mean........................			33	198	0.0

* See Equation 2 in the text.
† With 0.010 M fructose.
‡ Equilibrated for 4 hours.
§ Acetoacetic acid only determined. Not included in the mean.

C_8 can be determined (Leloir and Munoz, 1939). Hence the formation of all fatty acids from formic to octanoic can be excluded. (3) The respiratory quotient was low (mean 0.38). If the hypothesis of successive β oxidation is correct, there should have been large amounts of acetic acid formed (approximately 6 times the ketone formation). There was none whatever. This evidence is strongly corroborative of our previous conclusion that the chief mechanism of fatty acid oxidation in the liver is that of multiple alternate oxidation. Successive β oxidation or ω oxidation, if it occurs at all, is relatively insignificant.

The possibility that acetic acid formed and was *completely*

oxidized in the liver is ruled out by the value of the respiratory quotient which in that case should have been 0.65 ($C_{16}H_{32}O_2$ + $18.5O_2$ = $C_4H_8O_3$ + $12CO_2$ + $12H_2O$).

Molecular Ratio in Liver of Increase of Ketone Bodies to Decrease of Fatty Acids

Certain difficulties in this experiment should be pointed out. (1) The ideal preparation of liver slices would be one in which the ketone formation is high and the total fatty acids low. It was difficult to obtain livers from depancreatized cats in which this was so. When the ketone formation was large, the initial and final total fatty acids were so great that the measurement of the small difference was subject to such large errors that these experiments had to be discarded. Attempts to increase the subsequent ketone formation of the slices by daily feeding of thyroid gland for 2 weeks before the pancreatectomy were unsuccessful. Injection of dinitro-o-cresol 1 hour before the experiment to increase the total metabolism also failed to increase the rate of ketone body formation *in vitro*. (2) Phlorhizinized normal rats had low total liver fats, but the ketone formation was not particularly high, contrary to our hopes. (3) The known uneven distribution of total fats throughout the liver would be expected to contribute to the discordance of the small differences. However, by our method of sampling (see above) we sought to overcome this difficulty. (4) The method for the determination of fatty acid is good for about ±1 per cent. In looking for small differences (10 to 20 microequivalents per gm. of liver) between initial and final fatty acids, the total fatty acids would have to be <100 to 300 microequivalents per gm. of liver in order to keep the error of the differences at ±10 to 30 per cent.

We were successful in obtaining a series of experiments which from a technical point of view we considered reliable. The data in Table II show the following. (1) In all cases but one ketone body formation was accompanied by a decrease of total fatty acids. (2) In each experiment the replicate decreases of fatty acids are as concordant (±30 per cent) as could be expected from the nature of the experiment. (3) In all cases the ratio of ketone body increase to fatty acid decrease is >1 and the mean value is 3.3 ± 0.7.

TABLE II

Increase in Ketone Bodies and Decrease in Fatty Acids in Liver Slices from Depancreatized Cats and Phlorhizinized Cats and Rats

$t = 38°$; average sample 200 mg.; equilibrated 2.0 hours in phosphate-saline buffer at pH 7.2.   No supplements.

Animal No.	O₂ uptake, per gm. per 2 hrs.	R.Q.	Increase of ketones, per gm. per 2 hrs.	Initial fatty acids, per gm.	Change per gm. per 2 hrs.	Mean ratio, increased ketone to decreased fatty acids
				Diabetic cats		
	micromoles		micromoles	microequivalents	microequivalents	
Cat 121	175	0.55		202	−9	
	182	0.55		209	−25	
	168	0.53		201	−13	
	163	0.52		192	−30	
	179	0.56	30			
	171	0.52	21			
Mean.....			26 ± 5.6		−19 ± 6.0	1.4 ± 0.4
Cat 122B	166	0.25	69			
	179	0.29		290	−20	
	166	0.33		284	−12	
	164	0.35		253	+1	
Mean ..			69		−11 ± 6.5	6.3 ± 3.7
Cat 139A	164	0.29		145	−35	
	168	0.28		124	−19	
	172	0.29		139	−26	
	169	0.27	54			
	166	0.28	50			
Mean .			52 ± 3		−26 ± 4.7	2.0 ± 0.4
Cat 139B	182	0.32	70			
	178	0.31	58			
	184	0.27		346	−22	
	187	0.32		363	−41	
Mean			64 ± 6		−32 ± 10	2.0 ± 0.6

TABLE II—*Concluded*

Animal No.	O₂ uptake, per gm. per 2 hrs.	R.Q.	Increase of ketones, per gm. per 2 hrs.	Initial fatty acids, per gm.	Change per gm. per 2 hrs.	Mean ratio, increased ketone to decreased fatty acids

O_2 uptake, R.Q., Increase of ketones, Initial fatty acids, Change per gm., Mean ratio columns:

Animal No.	O_2 uptake, per gm. per 2 hrs.	R.Q.	Increase of ketones, per gm. per 2 hrs.	Initial fatty acids, per gm.	Change per gm. per 2 hrs.	Mean ratio, increased ketone to decreased fatty acids
	Normal fasted phlorhizinized rats					
	micromoles		*micromoles*	*microequivalents*	*microequivalents*	
144B	159	0.49	43			
	155	0.33	40			
	157	0.44		149	−21	
	156	0.33		142	−13	
Mean.................			41 ± 2		−17 ± 4.0	2.3 ± 0.6
144D	120	0.40	46			
	120	0.40		160	−17	
	120	0.16		167	−23	
Mean.................			46		−20 ± 3	2.3 ± 0.4
	Normal fasted phlorhizinized cats					
151A	152	0.33		135	−7	
	161	0.34	50			
	165	0.34		143	−13	
	161	0.33	50			
	154	0.33		139	−12	
	149	0.33	46			
	136	0.37		142	−4	
	141	0.38	43			
Mean............. ...			47 ± 2		−9 ± 2.5	5.2 ± 1.5
151B	124	0.29		260	−10	
	130	0.34	41			
	135	0.41		275	−10	
	137	0.34	48			
	125	0.34		254	−8	
	132	0.34	39			
Mean.................			43 ± 3		−9 ± 1	4.8 ± 0.6
Mean of all						3.3 ± 0.7

Our conclusion from the value of this ratio is that approximately 4 molecules of ketone body are formed by the partial oxidation of 1 fatty acid molecule in the liver. This is in accordance with the multiple alternate oxidation hypothesis and contrary to the successive β oxidation hypothesis. However, the mean value of 3.3 ± 0.7, while not significantly different from 4, the approximate value expected by the former hypothesis, might conceivably indicate that some oxidative reaction other than ketone body formation is occurring in the fatty acid catabolism of the liver. An oxidative process resulting in the formation of products other than ketone bodies, carbon dioxide, formic to octanoic acids, or carbohydrates would have to be supposed. Intermediary fatty acids with $C > 6$ must also be excluded. The fraction of total fatty acids so oxidized would be $(4 - 3.3 \pm 0.7)/4 = 0.2 \pm 0.04$. In other words, about 20 per cent of the total fatty acid decrease would have been brought about by such a process. It is difficult to conceive what this hypothetical product could be in view of the evidence already discussed which supports the multiple alternate oxidation hypothesis, particularly that on the respiratory balance of liver slices from diabetic cats. We are more inclined to attribute the discrepancy between the observed and the expected ratios to the totality of experimental errors inherent in the experiment.

SUMMARY

1. In liver slices from depancreatized cats, which when equilibrated *in vitro* produced abundant ketone bodies by partial oxidation of fatty acids, there was found no formation of acetic or other steam-volatile acids. This is corroborative evidence for the hypothesis of multiple alternate oxidation and against the hypothesis of successive β oxidation.

2. In similar slices from depancreatized cats and phlorhizinized rats and cats the ratio of ketone body increase to fatty acid decrease was found to be 3.3 ± 0.7. The significance of this with respect to fatty acid catabolism of the liver is discussed.

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CARBON SUBOXIDE AND PROTEINS

I. THE NATURE OF THE REACTION*

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With the advance of knowledge regarding the reactions of proteins, the significance of the number and distribution of polar groups within the molecule has become ever more appreciated. Since most of the polar groups are contributed by the side chains of dibasic and dicarboxylic amino acids, and of tyrosine, these groups would extend from a chain or stand up from the surface created by any orderly arrangement of chains, thus contributing a definite pattern to the protein. This pattern would to a great extent characterize the chemical and physiological behavior of the molecule. Of particular interest in this respect are the proteins having physiological action, such as enzymes and antibodies. The specificity of the former group is certainly dependent upon such a pattern, and a substrate must have a suitable pattern itself before union and subsequent action can take place. This is well illustrated by the recent work of Bergmann on the specificity requirements of substrates for the proteolytic enzymes.

Polar groups within proteins have been covered by suitable reagents, and the activity of the derived proteins studied. Thus Herriott and Northrop (1) have illustrated the importance of the phenolic hydroxyl of tyrosine for pepsin activity by acetylating this group with ketene. The same reagent has been applied to insulin by Stern and White (2). Hopkins and Wormall (3) after employing phenyl isocyanate have observed marked alteration of the specificity of serum globulin as an antigen. The reaction with insulin was also studied (4). These reagents have been more successful than others, as they are effective at low temperatures

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and at neutral or nearly neutral reactions, thus having a minimum denaturant effect on the proteins employed. They have in common the fact that they cover polar groups, removing charges from the protein pattern.

It has been the purpose of this research to develop reagents capable of replacing the positive charge of a basic group by the negative charge of a carboxyl group, and *vice versa*. With such reagents it should be possible to reverse the charge of certain polar groups on the protein surface. Carbon suboxide, C_3O_2, has been found to be a useful reagent for performing transformations of this type.

Carbon suboxide is considered to have the structure $O\!=\!C\!=\!C\!=\!C\!=\!O$, thus containing two active groups similar to that of ketene, $H_2C\!=\!C\!=\!O$. Its chemistry has been recently reviewed by Ryerson and Kobe (5). Its reactions with water to form malonic acid and with organic amines to give diamides of malonic acid are characteristic. Pauw (6) allowed C_3O_2 to react with amino acid esters in ether solution, and was able to isolate the malonyldiamides of the esters. Reactions of C_3O_2 with amino acids or amines in aqueous solutions had not, however, been investigated.

The reaction of C_3O_2 with a slightly alkaline aqueous solution of glycine was first studied to determine whether it would react by preference with water to form malonic acid or with the amino group of glycine to give either malonylmonoglycine or malonyl-diglycine. Isolation of as much as 75 per cent of the amino acid as malonyldiglycine (compound (III) in the accompanying scheme) has shown that C_3O_2 reacts much more rapidly with the basic amino group than with water. This conclusion is supported by the fact that we have not yet isolated from the reaction mixture malonylmonoglycine (II) which would be formed from the reaction

$$O\!=\!C\!=\!C\!=\!C\!=\!O + H_2NCH_2COOH \rightarrow$$

$$HOOCCH_2CONHCH_2COOH \ (II)$$

$$O\!=\!C\!=\!CHCONHCH_2COOH \quad \overset{(I)}{\nearrow}$$

$$\searrow \quad \overset{CONHCH_2COOH}{\diagup}$$

$$CH_2 \qquad \qquad (III)$$

$$\diagdown \quad CONHCH_2COOH$$
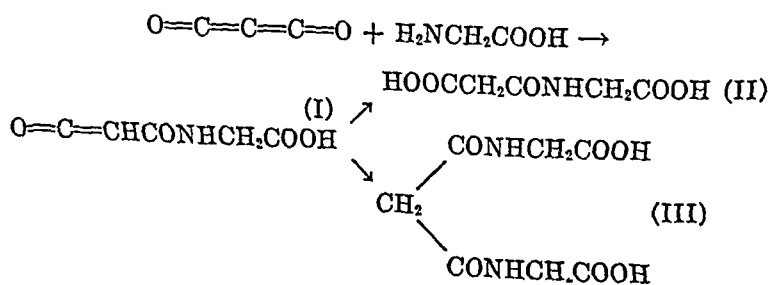
of the hypothetical intermediate (I) with a molecule of water. There is, of course, some malonic acid formed. The course of the reaction with glycine may be tentatively described in the accompanying scheme.

The behavior of C_3O_2 with solutions of glycine in more acid solutions is presented in Table I. These figures illustrate that amide formation occurs as low as pH 3, thus making the reagent available for use with proteins at any desired pH.

In addition to the reaction of C_3O_2 in water with the α-amino group of amino acids, it has been found (7) that it forms diamides of the ϵ-amino group of lysine and esters of the phenolic hydroxyl group of tyrosine.

<div align="center">TABLE I</div>

Reaction of C_3O_2 with Aqueous Solutions of Glycine at Varying pH

1 ml. portions of a 0.4 M C_3O_2-toluene solution were added to 10.9 ml. solutions containing 0.5 mM of glycine in 0.5 M buffers of appropriate pH. The original amino nitrogen concentration was 0.74 mg. per ml. The final amino nitrogen values have been corrected for the nitrogen evolved from the total malonic acid equivalent to the C_3O_2 taken. The "glycine coupled" is equal to the per cent of the original amino nitrogen which was lost during the reaction.

	pH 3	pH 5	pH 7	pH 8	pH 9
Final NH_2-N, *mg. per ml.*...	0.51	0.50	0.49	0.39	0.11
Glycine coupled, %	31	33	34	47	85

With proteins in aqueous solutions we should therefore anticipate the reaction of C_3O_2 with the terminal α-amino groups of polypeptide chains, with the ϵ-amino groups of lysine residues, and also with the phenolic hydroxyls of tyrosine. We should also expect that the intermediate corresponding to (I), and containing the half reacted C_3O_2 molecule, should react with water to form the malonylmonoamide similar to (II). The relative scarcity of amino groups, their fixed positions within the great molecule, and the low mobility of the protein molecule itself lead to this belief. The reaction within the protein could then be represented as

$$\boxed{P} \rightarrow \boxed{P}$$
$$\underset{NH_3^+}{|} \qquad \underset{NH \cdot COCH_2COO^-}{|}$$
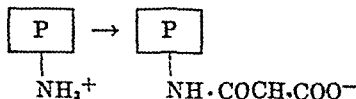
The conversion of a positive to a negative polar group in the same position, except for the insertion of the 3-C chain, would thus be accomplished.

<center>EXPERIMENTAL</center>

Preparation of Carbon Suboxide—The procedure of Hurd and Pilgrim (8) was followed. Liquid diacetyltartaric anhydride was slowly introduced into a horizontal tube at 650°; the products of pyrolysis were separated by first condensing the acetic acid at 15°, and then the C_3O_2 in a trap at dry ice temperatures. The liquid C_3O_2 was allowed to stand at 0° for an hour before use to effect the removal of dissolved CO_2.

Malonyldiglycine—A solution of 1.5 gm. of glycine in 30 ml. of water was brought to pH 8.5 by the addition of 1 N KOH and cooled to 0°; then carbon suboxide was distilled in by gently warming an excess of the liquid reagent. During the period of addition, 45 to 60 minutes, the reaction mixture was kept at 0° and the pH maintained constant by the addition of 1 N KOH. After completion of the reaction the solution was acidified with dilute HCl and concentrated in a vacuum. Crystallization, which had already begun at this point, was completed by allowing the solution to stand at 5° overnight. The product, 1.5 gm., consisted of clusters of monoclinic prisms, which were recrystallized from methyl alcohol and water or water alone. It melted at 236° with decomposition, having turned yellow 10° below this point.

Analysis—$C_7H_{10}N_2O_6$. Theory. C 38.5, H 4.6, N 12.8
Found.[1] " 38.6, " 4.7, " 12.8

Malonic acid was found in the residue after further concentration.

Malonyldiglycine from Diethyl Ester—Malonyldiglycine ethyl ester was prepared by the reaction of carbon suboxide with glycine ethyl ester in ether solution (6). The melting point was 107°, in agreement with Pauw. This was hydrolyzed by solution in water, addition of HCl to constant boiling, and concentration in a vacuum at 60–70°. The product formed monoclinic crystals, and after recrystallization from water melted at 235–236°, behaving exactly as did the product prepared from glycine in aqueous solution.

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[1] The carbon and hydrogen analysis was made by Miss L. S. Green.

Addition of C_3O_2 to Protein—For the addition of C_3O_2 the protein solution was placed in a slender cylindrical vessel surrounded with ice. The gas was introduced through a small sintered glass filter in the bottom of this vessel by gently warming several ml. of the liquid reagent. During the reaction the solution was stirred rapidly, and 0.3 N NaOH added from a burette at a point near the vanes of the stirrer. Alkali addition and C_3O_2 distillation were carefully regulated so that the pH remained at the desired value, a glass electrode being mounted in the vessel for this purpose.

Owing to the extended dilution of the reaction liquid by added base, a concentrated protein solution was desirable at the beginning of experiments. The time required for the addition of C_3O_2 varied from 2 to 6 hours, dependent, of course, upon the quantities used. Slow addition appeared to give less denaturation.

Analysis—It was at first hoped that amino nitrogen content could be used as a measure of the extent of reaction of C_3O_2 with free amino groups. A few experiments led to the observation that malonic acid yielded large amounts of gas in the Van Slyke amino nitrogen determination, as if the malonic acid contained about 5 per cent of amino nitrogen. If enough potassium iodide were added with the acetic acid to maintain the color of iodine throughout the period of shaking, the formation of nitrogen by the malonic acid was prevented. With proteins, however, a yellow color in the presence of nitrous acid was not always evidence of free iodine. In addition it was found that the amino nitrogen values obtained with egg albumin preparations containing malonyl groups were considerably lower when iodine was used in the analysis, whereas untreated egg albumin gave practically the same amino nitrogen whether or not iodine was used. This result indicated that both free and combined malonic acid interfered with the amino nitrogen determination.

The following procedure was found satisfactory for separating the treated protein from uncombined malonic acid. After the reaction solution had stood about half an hour to insure complete decomposition of C_3O_2, the pH was adjusted to 4.6 and the precipitation completed by the addition of 1 to 2 volumes of concentrated salt solution. The precipitate was filtered off by suction, washed once with 20 per cent salt solution, and redissolved in water. After a second precipitation, the protein was redis-

solved in water and dialyzed for 3 days against distilled water. The complete removal of malonic acid is established by the data below, in moles of malonic acid per gm. atom of nitrogen.

Experiment No.	Initial	First ppt.	Second ppt.
1	0.88	0.006	0.001
2	0.46	0.004	0.001

Van Slyke amino nitrogen determinations on treated proteins thus purified were found to be satisfactory as long as excess iodine

<div align="center">

TABLE II

Crystalline Egg Albumin and Carbon Suboxide

</div>

The albumin had been twice recrystallized. The original ratio of amino to total nitrogen = 0.044.

Experiment No.	1	2a	2b	2c	3	4a	4b
pH	8.0	6.9	6.9	6.9	7.0	6.2	6.2
C_3O_2 per gm. atom N, *mole*	0.6*	0.16*	0.42*	0.48*			0.18
Combined malonyl per gm. atom N, *mole*	0.031	0.0124 0.0161	0.043 0.044 0.041	0.029 0.033 0.031	0.022	0.007	0.023
Ratio, final amino to total N	0.007	0.025	0.010	0.016 0.010	0.029	0.035	0.025
Sum of 2 figures above	0.038	0.039	0.053	0.044	0.051	0.042	0.048

* From base required to keep pH constant; too high on account of some CO_2 in C_3O_2.

was present, and shaking was continued for 15 minutes. It is, however, difficult to determine very small quantities of amino nitrogen in proteins.

In view of this fact, a method was developed for the direct determination of combined malonic acid. This is described in the following communication (9). This determination, in addition to giving more reliable values than can be obtained from estimating the disappearance of amino nitrogen, measures non-amide malonyl groups.

The experimental data for the runs made with egg albumin, horse serum albumin, and chymotrypsin are presented in Tables II to IV, respectively.

Electrometric Titration—The titration assembly consisted of an enclosed rotating cup of 25 ml. capacity in which were immersed the non-rotating glass electrode assembly and tip of a micro-

TABLE III

Serum Albumin and Carbon Suboxide

Carbohydrate-free crystalline horse serum albumin of McMeekin (10). The original ratio of amino to total nitrogen = 0.0813. pH = 7.5, all experiments.

Experiment No	1a	1b	1c	1d	2
C_3O_2 per gm. atom N, *mole*	0.152*	0.344*	0.61*	1.03*	0.341
Combined malonyl per gm. atom N, *mole*	0.024	0.047	0.059	0.066	0.045† 0.046‡
Ratio, final amino to total N	0.072	0.045	0.041	0.028	0.047 0.052
Sum of 2 figures above	0.096	0.092	0.100	0.094	0.095

* Estimated by measuring the difference in nitrogen released in the Van Slyke determination in the presence and absence of excess iodine.

† Analysis by Miss Ann H. Tracy.

‡ Figure obtained from the titration curve; see the text.

TABLE IV

Chymotrypsin and Carbon Suboxide

Crystalline preparation of Northrop (11). pH = 7.5. The original ratio of amino to total nitrogen = 0.063.

Experiment No	1a	1b	1c	1d	Original enzyme
C_3O_2 per gm. atom N, *mole*	0.343	1 09	2.47	6.77	
Combined malonyl per gm. atom N, *mole*	0 018	0.031	0.041	0.047	
Ratio, final amino to total N		0.044	0 037	0.037	0.063
Sum of 2 figures above		0.075	0.078	0 084	
Proteolytic* activity	0.096	0.050	0.040	0.029	0.160

* The increase in NH_2 to total nitrogen after 42 hours in a digest containing per 10 ml., 37 mg. of egg albumin nitrogen, 4 mg. of enzyme nitrogen, 1.3 ml. of 0.66 N Na_2HPO_4 buffer, pH 8 2.

burette. The current drawn from this was amplified by a two-stage amplifier and compared with that from a Leeds and Northrup type K potentiometer, with a microammeter as null indicator.

Titrations were of the continuous type; a quantity of protein solution was placed in the vessel and E.M.F. determinations made after the successive addition of fractional ml. quantities of 0.5 N KOH or HCl, 2 minutes of stirring being allowed for equilibrium. Concentrations were such that 30 mg. of protein nitrogen were present in a 12 ml. total volume.

Fig. 1 gives the titration curves for malonyl serum albumin (Table III, Experiment 2) and normal serum albumin. The treated protein had been purified in the usual way by precipitation and then by dialysis in a rotating cellophane membrane at 0°
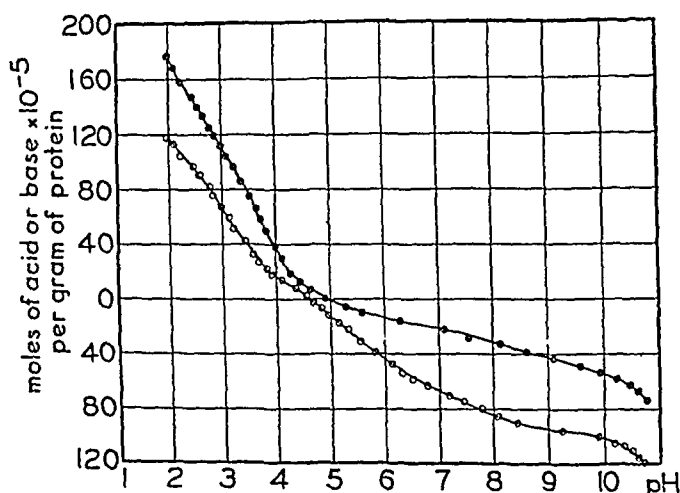
FIG. 1. Titration curves for horse serum albumin, and malonyl serum albumin. ○ represents malonyl serum albumin (Table III, Experiment 2); ◐, normal serum albumin.

for 1 day against water, 1 day against dilute NaCl solution, and finally 2 days against water. The normal serum albumin, prepared according to McMeekin (10), had been recrystallized four times and dialyzed in a similar manner for 5 days.

The values of combined acid and base were calculated in the usual way from the E.M.F. determinations. Activity coefficients for the H+ were taken from Lewis and Randall (12) and a value of 13.77 for pK_w was used. The ionic strength was considered to be that of the NaOH or HCl present, that due to the protein being neglected. 16.1 per cent nitrogen content was accepted for both proteins (10).

DISCUSSION

The analytical data for C_3O_2-treated proteins (Tables II to IV) offer strong support for the interpretation that this reagent converts free amino groups of proteins into corresponding malonyl derivatives.

Although no complete amino acid analysis of the crystalline horse serum albumin of McMeekin is yet available, earlier values for serum albumin (13) give a lysine content equivalent to 0.0774 mole per gm. atom of nitrogen. Lysine contributes the major portion of free amino groups. To its value must be added whatever amino nitrogen is present at the ends of polypeptide chains. Our figure for the total amino nitrogen per gm. atom of nitrogen, 0.0813, is ample to cover both. If C_3O_2 reacted only with amino groups, the sum of the combined malonic acid and the amino nitrogen per gm. atom of nitrogen would be constant and equal to the original value of amino nitrogen. Our figures for this sum, 0.096 ± 0.004, are remarkably constant (bottom row of Table III), but are consistently about 20 per cent higher than the original amino nitrogen value, suggesting that malonyl is being introduced at some other point. C_3O_2 might react with the phenolic groups of tyrosine, but less rapidly than with amino groups if it behaves like ketene, which was investigated by Herriott and Northrop. The fact that these figures do not increase proportionally with the amount of C_3O_2 reagent added indicates that this second group is about as reactive as an amino group. The tyrosine content, 0.023 mole per gm. atom of nitrogen (14), added to the lysine content, 0.0774 mole, equals 0.1004. One terminal amino group per molecule of 73,000 molecular weight and 16.1 per cent nitrogen content would contribute 0.0012 to this figure. Several such groups would not be excluded by our data.

The titration curve for malonyl serum albumin, Experiment 2 of Table III, is shown in Fig. 1.[2] There are approximately

[2] Through the kindness of Professor Edwin J. Cohn of the Harvard Medical School, we have been able to compare our curve for McMeekin's carbohydrate-free serum albumin with one obtained in his laboratory by the use of the hydrogen electrode and discontinous titration. There is good agreement between pH 3.5 and 9.5, but our curve has less tendency to flatten out at extreme acid and alkaline concentrations, particularly between pH 2 and 3. We believe that these differences are due to devia-

50×10^{-5} additional base-combining groups per gm. of treated protein at pH 10.7, which is equivalent to 0.0435 mole of malonic acid per gm. atom of nitrogen, assuming that only one carboxyl group is free.   Likewise there are 56×10^{-5} less acid-combining groups at pH 2.2 in the same protein, or 0.0487 mole per gm. atom of nitrogen.   The average of these agrees well with the analytical figure for combined malonic acid, 0.045.   Therefore each molecule of C_3O_2 which reacts covers up a basic or acid-combining group and introduces an additional acidic or base-combining group.   We believe the only interpretation is that which we have already suggested.   From these data it would appear that there is very little diamide formation, either intra- or intermolecular.

Similar calculations can be made for malonyl egg albumin, Table II, but the data here are not as satisfactory, this being the first protein studied and that upon which the experimental procedures were worked out.   Both it and malonylchymotrypsin, however, support the general conclusions drawn from our study of malonyl serum albumin.

Most of the malonyl proteins are insoluble in dilute salt solutions from pH 4.6 to 3.5.   This may be caused by a stronger isoelectric effect in the treated protein or by limited denaturation. The solubility appears to be lower after reactions under pH 7.0. Both the removal of acid-combining groups and the introduction of carboxyls would decrease solubility in weakly acid solution. The proteolytic activity of malonylchymotrypsin is found to be inversely proportional to the amount of malonic acid introduced (Table IV), which could be the result of denaturation or of changed enzymic specificity.

We are now investigating the specificity requirements of C_3O_2-treated enzymes with both normal proteins and synthetic substrates.   A study of the digestion of malonyl proteins by normal and malonyl enzymes is also in progress.

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tions of the glass electrode and plan to repeat the titrations using a hydrogen electrode assembly.   If they do result from our titration procedure, any correction found to be necessary would apply equally to the curves for both normal and malonyl proteins without affecting the conclusions drawn here.

SUMMARY

A reaction is described whereby it is possible to convert certain acid-combining or basic groups within protein molecules into groups of an opposite charge.

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CARBON SUBOXIDE AND PROTEINS

II. THE DETERMINATION OF MALONIC ACID*

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The procedure presented here has been developed to permit quantitative study of the reaction of carbon suboxide with proteins in aqueous solution (1). To liberate malonic acid from the malonyl groups thus introduced, alkaline hydrolysis was employed. This malonic acid was extracted from the acidified hydrolysate by ether and then transferred to water. Aliquots of the resulting aqueous solution were titrated with ceric sulfate (Willard and Young (2)), one directly and a second after heating at 140° in acid solution. This pyrolysis decomposes malonic acid into acetic acid and carbon dioxide, neither of which reduces ceric sulfate. The difference between the two titrations represents the malonic acid present; interference by other reducing material is thus avoided unless it too is destroyed by pyrolysis.

EXPERIMENTAL

Hydrolysis of Proteins—Malonic acid was found to be sufficiently stable to permit hydrolysis of proteins by alkali at 100° without loss. Acid hydrolysis under similar conditions resulted in considerable destruction (Table I). Pure malonic acid could be satisfactorily recovered as acetic acid by steam distillation following phosphoric acid hydrolysis in a sealed tube at 120° for 24 hours. However, normal proteins yielded acids volatile with steam under the same conditions (Table II).

Analysis—In view of these results the determination of malonic acid by oxidimetry was adopted. The procedure as finally accepted follows.

A quantity of protein containing 2 to 25 mg. of malonic acid is dissolved in a small amount of water and sufficient saturated NaOH added to make the protein solution 2 N in NaOH, after having 1 milliequivalent of alkali present for each 100 mg. of protein. The tube is sealed, and heated at 100° for 24 hours. Concentrated HCl equivalent to the NaOH and enough additional to make the solution about 2 N in strength is then added.   The

TABLE I

Stability of Malonic Acid to Hydrolyzing Agents

Agent	Temperature	Time	Malonic acid recovery
	°C.	hrs.	per cent
6 N HCl	120	12	0
6 " "	102	18	46
0.5 " NaOH	100	20	100
2 " "	100	20	99
3 " "	100	20	99
5 " "	100	20	102

TABLE II

Determination of Malonic Acid by Acetic Acid Released upon Decomposition
Hydrolysis at 120° for 24 hours by 20 per cent H_3PO_4, in a sealed tube.

Protein	Malonic acid added	Malonic acid recovered
	mg.	mg.
None...........................	10	9.84
"	10	9.96
"	10	9.79
0.4 gm. gelatin....................	None	4.96
0.4 " egg albumin....	"	3.08

solution is agitated thoroughly, washed into an extractor of. the Lormand type (3), and extracted with diethyl ether, 2 hours for each ml. of solution. The ether extract is transferred quantitatively to a small separatory funnel, and extracted with about 5 ml. of water containing 0.5 milliequivalent of NaOH. The aqueous extract is run into a 10 ml. volumetric flask, and the ether layer washed with three or four small portions of water which are added to the flask.

This is then heated, first cautiously at 50°, then at 95° for 1 hour; after cooling, it is diluted to the mark.   One 4 ml. aliquot is introduced into a test-tube, with slightly more than an equivalent quantity of dilute H_2SO_4.   The tube is then sealed and heated 90 minutes at 140°, after which the contents are washed into a small Erlenmeyer flask.   A second 4 ml. aliquot is introduced into another Erlenmeyer flask of the same size.   Into each solution is measured 0.1 N ceric sulfate in 2 N H_2SO_4 considerably in

TABLE III

Apparent Malonic Acid Content of Egg Albumin

Preparation	Protein nitrogen	Malonic acid recovered
	mg.	*mg.*
Commercial	83	0.00
Crystalline	62	0.06
"	109	0.02

TABLE IV

Recovery of Malonic Acid Added to Egg Albumin

Protein nitrogen	Malonic acid added	Malonic acid recovered
mg.	*mg.*	*mg.*
47	8.90	8.68
47	8.90	9.10
47	8.90	8.63
31	4.45	4.54
31	4.45	4.39
31	4.45	4.35
53	8.90	8.75
53	8.90	8.91
53	8.90	8.67

excess of the malonic acid believed present, followed by an equal volume of 18 N sulfuric acid.   Water is added to bring the volume to about 4 times that of the ceric sulfate.   The two flasks are set side by side in an oven or bath at 90° and heated 30 minutes. Upon cooling to 30° they are titrated with ferrous ammonium sulfate solution (0.1 N in 0.3 N H_2SO_4), with o-phenanthroline ferrous sulfate as indicator (4).   The difference in reducing capacity of the two solutions is due to the destruction of malonic acid.   The

ferrous ammonium sulfate is standardized against the ceric sulfate solution, which in turn is standardized with sodium oxalate. 1 milliequivalent of ceric sulfate is equivalent to 15.63 mg. of malonic acid (2).

This method has been applied to egg albumin preparations to which no malonic acid and known quantities of malonic acid had been added. From egg albumin itself, malonic acid recoveries were negligible (Table III); from preparations to which malonic acid had been added they represented 97 to 102 per cent of that present (Table IV).

No attempt was made to ascertain the most gentle hydrolytic conditions for the liberation of malonic acid, as experiments had shown that 0.5 N and 4 N sodium hydroxide were equally effective. The ether extraction proceeded more smoothly when extended hydrolysis had occurred.

SUMMARY

A method of analysis for malonic acid present in malonyl proteins is presented.

This method should be equally satisfactory for either free or combined malonic acid in other biological materials.

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CARBON SUBOXIDE AND PROTEINS

III. THE REACTION OF CARBON SUBOXIDE WITH AMINO ACIDS

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In Paper I of this series (1) there was reported the reaction of carbon suboxide, C_3O_2, with an aqueous solution of glycine to give malonyldiglycine. The present communication describes further studies of the behavior of this reagent with amino acids in the presence of water.

Like glycine, lysine adds to C_3O_2 through its α-amino group if the ϵ-amino is protected. Thus there was obtained from ϵ-benzoyl-*dl*-lysine the expected malonyldi-(ϵ-benzoyl-*dl*-lysine)α-amide. This behavior can be accepted as typical of all primary α-amino groups, and indicates that the free terminal amino groups in proteins react similarly.

The ϵ-amino group of lysine also gives well defined crystalline products if the α group is protected. From α-benzoyl-*l*-lysine there was obtained malonyldi-(α-benzoyl-*l*-lysine)ϵ-amide, which formed equally well in solutions of pH 8.0 or 6.0. The loss of amino nitrogen by proteins on exposure to C_3O_2 has already been attributed to the reaction of this group in the lysine residue.

There is, however, in malonyl proteins more combined malonic acid than can be accounted for by amide formation alone (1). This led to the investigation of the behavior of C_3O_2 with suitable derivatives of tyrosine, an amino acid which is known to be acetylated at its phenolic hydroxyl by ketene (2). Both the diester, di-(N-carbobenzoxy-*l*-tyrosyl) malonate and the mono-ester, mono-(N-carbobenzoxy-*l*-tyrosyl) malonate were found to be present after the addition of 1.5 molar equivalents of C_3O_2 to a slightly alkaline solution of carbobenzoxytyrosine. The yields,

90.5 per cent of the diester and 2.4 per cent of the monoester, indicate not only that C_3O_2 reacts readily in aqueous solution with the phenolic hydroxyl, but also that the half reacted intermediate has a greater preference for such a group than for water. The scheme which was proposed in an earlier paper (1) may now be accepted in its complete form.

The low melting point of mono-(N-carbobenzoxy-*l*-tyrosyl) malonate (52°) may well explain our lack of success in isolating malonylglycinemonoamide from the reaction of C_3O_2 with aqueous glycine. A correspondingly low melting point for this compound would make its isolation from the by-products of the reaction difficult. Oils have been obtained from the more soluble fractions,

$$O=C=C=C=O + HOC_6H_4CH_2CHCOOH \rightarrow$$
$$|$$
$$NHCbzo$$

$$(II)\ HOOCCH_2COOC_6H_4CH_2CHCOOH$$
$$2.4\%\qquad\qquad\qquad |$$
$$NHCbzo$$

$$O=C=CHCOOC_6H_4CH_2CHCOOH$$
$$(I)\qquad\qquad |$$
$$NHCbzo$$

$$COOC_6H_4CH_2CHCOOH$$
$$/\qquad\qquad\qquad\quad |$$
$$(III)\ CH_2\qquad\qquad NHCbzo$$
$$\backslash$$
$$COOC_6H_4CH_2CHCOOH$$
$$|$$
$$NHCbzo$$
$$90.5\%$$
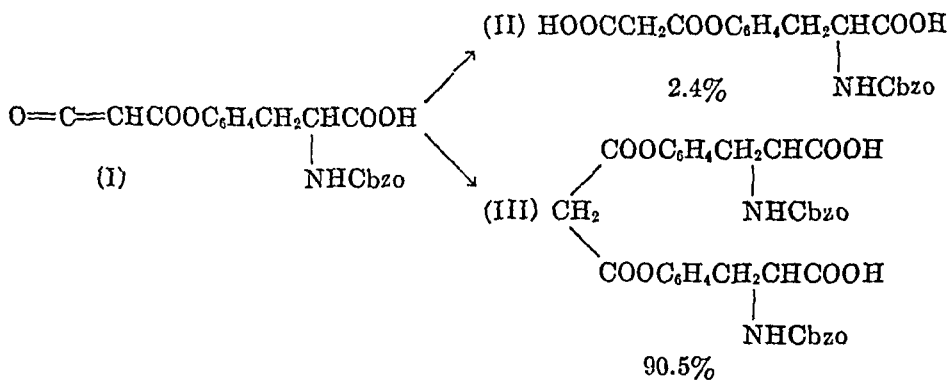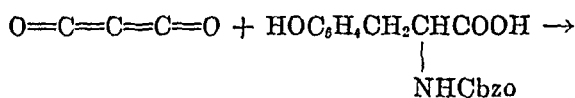
but no crystalline product other than malonic acid has been recovered.

The isolation of the monoester is also significant for it proves that the half reacted intermediate (I) may combine further with either water or a suitable amino acid residue. The first course is preferred when the reactive residue is within a protein molecule, the latter when it is in a mobile low molecular amino acid derivative and when other such molecules are available.

The reaction of C_3O_2 with the phenolic hydroxyl group appears to proceed at approximately the same rate as with the primary amino group. Identical amounts of this reagent in toluene solution were added to equimolar quantities of carbobenzoxy-*l*-tyrosine

and glycine in two exactly parallel experiments. Of the carbo-benzoxytyrosine, 93 per cent was later accounted for as either the di- or monoester; of the glycine 67 per cent was found as the diamide, there being no recovery of the monoamide.

Suitable derivatives of serine, arginine, and histidine have been investigated to determine whether C_3O_2 reacts with the functional groups of their side chains. Under the conditions we employ, no evidence for reaction was obtained. It was interesting to observe that C_3O_2 reacted almost quantitatively with dilute aqueous ammonium hydroxide to form malonamide.

Cahill and coworkers (3) have reported the racemizing action of ketene in slightly acid solution. We have not observed any significant alteration of optical activity after addition of C_3O_2 to solutions of amino acid derivatives of pH 6. It is possible, however, that C_3O_2, when added to weakly acidic solutions of proteins, racemizes certain of the amino acid residues at the carboxyl ends of protein chains, thereby causing, through denaturation, the increased insolubility of malonyl proteins formed under slightly acidic conditions.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Lysine*</div>

Malonyldi-(ε-Benzoyl-dl-Lysine)α-Amide—ε-Benzoyl-*dl*-lysine was available as an intermediate in the synthesis of *dl*-lysine (4). An aqueous solution of 1.2 gm. of it was treated with C_3O_2 at a pH of 8.0, the molar ratio of C_3O_2 to amino acid being 2.0. From the reaction solution was obtained 0.8 gm. of long monoclinic crystals which decomposed at 239–240°.

<div align="center">

$C_{23}H_{31}O_5N_4$. Calculated. C 61.2, H 6.4, N 9.9
Found. " 60.9, " 6.6, " 10.2

</div>

α-Benzoyl-ε-Carbobenzoxy-l-Lysine—Benzoylation of ε-carbo-benzoxy-*l*-lysine (5) in aqueous solution gave the corresponding α-benzoyl derivative which first formed as an oil but was later crystallized to give microscopic prisms melting at 107°.

<div align="center">

$C_{21}H_{24}O_5N_2$. Calculated. C 65.6, H 6.3, N 7.3
Found. " 65.7, " 6.7, " 7.3

</div>

α-Benzoyl-l-Lysine—The α-benzoyl-ε-carbobenzoxy-*l*-lysine from 4.0 gm. of ε-carbobenzoxy-*l*-lysine, as an oil, was hydrogenated in aqueous methyl alcohol with palladium black as catalyst. The product was crystallized from water, forming thick needles which melted with sintering at 250°.   1.1 gm. were obtained.   The corresponding *dl*-lysine derivative has been reported by Greenstein (6).

$$[\alpha]^{25} = +21.6° \text{ (in water, containing 1 equivalent of NaOH)}$$

Malonyldi-(α-Benzoyl-l-Lysine)ε-Amide—C_3O_2 was slowly passed into a cold solution of 0.5 gm. of α-benzoyl-*l*-lysine in 30 ml. of 0.1 N borate buffer of pH 8.0.   During this addition 11 ml. of 1 N NaOH were required to keep the pH constant.   This corresponds to a molar ratio of C_3O_2 to amino acid derivative of 2.6. After acidification and concentration, 0.25 gm. of product, malonyldi-(α-benzoyl-*l*-lysine)ε-amide, was obtained.   This was recrystallized from water for analysis.   It melted at 267–268° with decomposition.

$C_{29}H_{36}O_8N_4$.   Calculated,   C 61.2, H 6.4; found, C 61.1, H 6.6

From a reaction between C_3O_2 and 0.6 gm. of α-benzoyl-*l*-lysine in 30 ml. of 0.1 N phosphate buffer of pH 6.0 was isolated 0.35 gm. of the same diamide.   The molar ratio of C_3O_2 to amino acid was 2.6 here also.   The product melted at 264–266°.   There was not enough for significant optical rotations to be made.   Found, C 61.0, H 6.5.

Tyrosine

Di-(N-Carbobenzoxy-l-Tyrosyl) Malonate—A solution of 2.0 gm. of N-carbobenzoxy-*l*-tyrosine (7) in 25 ml. of water was brought to a pH of 8.5 with several ml. of 1 N KOH and then shaken with 1.4 molar equivalents of C_3O_2 in toluene.   After a period of shaking 30 minutes at 0° and 60 minutes at room temperature, 10 ml. of 1 N KOH had been added to keep the pH approximately constant. The aqueous layer was then acidified with 1 N HCl upon which colorless rhombs of di-(N-carbobenzoxy-*l*-tyrosyl) malonate formed.   The product, 2.01 gm., was recrystallized from methyl

alcohol and water for analysis. It melted at 135° with decomposition.

$C_{37}H_{34}O_{12}N_2$. Calculated. C 63.6, H 4.9, N 4.0
Found.   " 63.4, " 5.2, " 3.9
Neutralization equivalent. Calculated, 349; found, 368

The alkaline hydrolysis of 2.0 gm. of di-(N-carbobenzoxy-*l*-tyrosyl) malonate was followed by the recovery of 1.5 gm. of N-carbobenzoxy-*l*-tyrosine and 0.2 gm. of malonic acid. These quantities represent 83 and 67 per cent, respectively, of the theoretical for the accepted structure.

Di-(N-Carbobenzoxy-l-Tyrosyl Methyl Ester) Malonate—Methylation of 0.5 gm. of di-(N-carbobenzoxy-*l*-tyrosyl) malonate with diazomethane gave 0.4 gm. of the corresponding ester, which after recrystallization from ether and methyl alcohol formed long prisms melting at 145° with decomposition.

$C_{39}H_{38}O_{12}N_2$. Calculated, C 64.4, H 5.3; found, C 64.4, H 5.3

Mono-(N-Carbobenzoxy-l-Tyrosyl) Malonate—The mother liquor from the preparation of di-(N-carbobenzoxy-*l*-tyrosyl) malonate above was concentrated in a vacuum to a volume of 5 ml. and cooled to 0°. 0.06 gm. of long prisms melting at 53° formed. These were recrystallized from hot water for analysis.

$C_{20}H_{19}O_8N$. Calculated. C 59.8, H 4.8, N 3.5
Found.   " 59.8, " 5.1, " 3.6

In order to make a comparison with the product from above, mono-(N-carbobenzoxy-*l*-tyrosyl) malonate was synthesized from malonyl monochloride and N-carbobenzoxy-*l*-tyrosine. 0.4 gm. of malonyl monochloride (8) was coupled at 0° with 1.0 gm. of carbobenzoxy-*l*-tyrosine in 20 ml. of water containing 8 ml. of 1 N NaOH. After a wash with chloroform, the reaction solution was made acid to Congo red with HCl and concentrated in a vacuum. The product, long prisms, weighed 0.4 gm. and melted at 52°, giving no depression in a mixed melting point determination with the monoester from C_3O_2. On standing it gradually turned brown. Found, C 59.7, H 5.0.

Arginine

α-p-Nitrobenzoyl-l-Arginine—Arginine monohydrochloride, 3.5 gm., was coupled with 3.7 gm. of *p*-nitrobenzoyl chloride in aqueous solution in the presence of excess potassium carbonate.   The product, 3.5 gm. of needles, was recrystallized from hot water for analysis.   It melted at 226–228° with decomposition.

$$C_{13}H_{17}O_5N_5. \quad \text{Calculated, C 48.3, H 5.3; found, C 48.3, H 5.3}$$
$$[\alpha]^{28} = -80.5°$$

α-p-Nitrobenzoyl-l-Arginine and C_3O_2—From a reaction between C_3O_2 and *α-p*-nitrobenzoyl-*l*-arginine at a pH of 8.5 and at 3° the starting product was recovered in a yield of 86 per cent.   The ratio of C_3O_2 to amino acid was 1.7.   The recovered product melted at 224–227°. $[\alpha]^{28} = -75.3°$.

Similarly an 81 per cent recovery of *α-p*-nitrobenzoyl-*l*-arginine was obtained when C_3O_2 was added at a pH of 6.0, the molar ratio being 2.04.   This product melted at 226–228°, and its optical activity remained the same, $[\alpha]^{28} = -80.8°$.

Histidine

α-N-Benzoyl-l-Histidine and C_3O_2—A solution of 1.0 gm. of *α*-N-benzoyl-*l*-histidine (9) in 25 cc. of 0.1 N borate buffer of pH 8.0 was treated in the usual way with 2.26 molar equivalents of C_3O_2.   From the reaction solution after acidification and concentration a 92 per cent recovery of unchanged starting material was obtained.   A mixture with the original melted at 247°.

Serine

N-p-Nitrobenzoyl-dl-Serine   and C_3O_2—N-*p*-Nitrobenzoyl-*dl*-serine was prepared according to Fischer and Jacobs (10).   A solution of 1.5 gm. of it in 30 ml. of 0.1 N borate buffer of pH 8.0 was treated at 0° with C_3O_2.   The molar ratio of the latter to the amino acid derivative was 1.3.   There was obtained an 87 per cent recovery of the starting material, which gave an unchanged melting point of 198–200° when mixed with the original.

Malonamide—An excess of C_3O_2 was passed into 2 per cent aqueous ammonium hydroxide at 0°.   On concentration of the reaction solution, colorless octahedral crystals of malonamide were deposited.   These melted at 169–170°.

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THE DETERMINATION OF CHLORIDE IN SINGLE ISOLATED MUSCLE FIBERS

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It is frequently assumed that the chloride in muscle is entirely outside the fibers and that the tissue spaces contain chloride in equilibrium with the plasma or other external fluid. Reasons for this assumption are reviewed by Fenn (1) who found tissue spaces of 14.7 per cent in frog muscle. Maurer (2) found that the extracellular fluid collected from frog muscle had the same chloride content as the plasma. Manery, Danielson, and Hastings (3) modified the theory to include connective tissue in the chloride space. Conway, Kane, Boyle, and O'Reilly (4) using a method based on rates of diffusion concluded that only 9 per cent of frog muscle is tissue space and that the remaining five-fourteenths of the chloride is inside the fibers. When muscle is soaked in Ringer's solution, the chloride space increases to 27 per cent but Conway *et al.* found the extracellular space to remain unchanged at 9 per cent.

It would seem likely that chloride contained in connective tissue and in any fibers which might have become permeable to chloride because of injury would diffuse out more slowly than chloride in the tissue spaces and therefore the data of Conway *et al.* do not prove the existence of chloride inside uninjured muscle fibers. It seemed desirable therefore to measure the chloride content of single isolated muscle fibers. Certain difficulties particularly in washing the fiber inevitably introduce some uncertainty into the results but it can be shown nevertheless that the figures are inconsistent with the theory of Conway *et al.* (4).

Methods

Chloride was determined by a modification of the ultramicromethod of Wigglesworth (5). From a waxed capillary pipette a

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standard volume (about 1 c.mm.) of $AgNO_3$ solution containing a little $Fe(NO_3)_3$ was delivered onto a waxed slide. From another pipette a similar drop of HNO_3 was added and the 2 drops were mixed with the sample. The resulting drop was covered with a micro bell jar and incubated to digest the tissue and coagulate the AgCl precipitate. The excess silver was then titrated with KCNS from a micro-Rehberg burette (6). The tip of the burette was immersed in the drop and stirring was accomplished by blowing a jet of air against one side of the drop, causing it to spin rapidly.

Ceresin wax was used and the waxed slides had slight depressions melted in the wax with a hot glass rod to hold the drop. Micro bell jars 7 mm. in diameter were coated with wax and just before the drop was covered the edge of the jar was heated to melt the wax. In this way evaporation of the drop during digestion was prevented. The slides containing usually 3 drops were incubated overnight at 37° or in some cases for 2 days at 30°. This treatment resulted in a clear light yellow drop with a very tough surface film which had to be broken before the drop could be stirred by an air jet. The silver solution contained about 0.02 M $AgNO_3$, 1 per cent $FeNO_3$, and 10 per cent HNO_3. The KCNS was about 0.01 M.

A micro-Rehberg burette was made from a broken 0–110° thermometer. One end was drawn out to make a delivery tip. The capillary at the other end was expanded to make a shallow cup. A cylindrical steel chamber 1.2 cm. in diameter and 2.5 cm. long was made with a concentric hole just large enough to take the thermometer tubing and 1.5 cm. long. A thin hole threaded to take a 2-56 screw continued through the longitudinal axis of the chamber and was fitted with a screw bearing a large knurled head. The steel chamber was cemented to the thermometer tube with De Khotinsky cement. The whole burette was filled with mercury and air bubbles were removed by repeated evacuations in a tube connected to a water pump. The screw was lubricated with vaseline and the burette was always kept inclined at about 15° to the horizontal with its tip under water when not in use. One turn of the screw moved the mercury thirty divisions of the scale, delivering about 1 c.mm. The volume delivered could be adjusted to within one-quarter of a division with a little practice.

KCNS solution was drawn into the burette by immersing the tip in the solution and turning the screw back until the mercury was just above the zero line. The mercury was adjusted exactly to zero and the tip was then washed off with 10 drops of distilled water and wiped with filter paper. The tip of the burette was placed inside the drop to be titrated. A little wax on the outside of the tip facilitates stirring of the drop.

The burette and KCNS solution were standardized by titrating a drop of 0.0055 M NaCl delivered from a waxed capillary pipette. The same pipette was then used to deliver a drop of 2.77 M NaCl which was titrated by the method of Keys (7). Assuming that the capillary pipette delivered equal volumes of the two concentrations of NaCl, it was possible to calculate the chloride equivalent of one division of the burette. The KCNS solution was standardized several times in this way during the course of the work and the concentration was assumed to have changed linearly with time between successive standardizations.

Single muscle fibers from the semitendinosus muscle of the frog *Rana pipiens* were kindly dissected out for this work by Dr. S. F. Street (Ramsey and Street (8)). The fibers were dissected out in Ringer's solution which was 0.114 M in chloride. They were mounted on platinum wire hooks attached to a glass rod so that the fiber was stretched approximately rest length parallel to the glass rod and about 5 mm. from it.

All fibers were tested for conduction after being mounted and were considered to be normal if they gave a conducted response to a short tetanizing current from a Harvard inductorium. Some fibers designated as non-conducting gave only local twitches. Some fibers were rendered permeable to chloride by immersion in 4 per cent formaldehyde in Ringer's solution for a few minutes and were then equilibrated overnight in Ringer's solution before being analyzed.

The diameter of the fiber while still under Ringer's solution was estimated at 3 to 5 points along its length by means of an ocular scale in a microscope with a low power objective. The magnification was calibrated by measuring the apparent diameter of a rod in Ringer's solution. The true diameter of the rod was obtained with a machinist's micrometer. Since the fibers showed some variation of diameter along their lengths, the average di-

ameter was taken to calculate the volume by the formula for the volume of a cylinder. Trial calculations with the more exact formula for the frustrum of a cone showed that this approximation introduced an error of less than 5 per cent. The length was es timated by comparison with a mm. scale, 0.5 mm. being left for attachment at each end. This approximate procedure was made necessary by the fact that the fiber shrinks while drying, so that the actual length cut off bore an unknown ratio to the length in Ringer's solution.

When the fiber was to be analyzed, the glass rod was lifted through the surface of the Ringer's solution first and the film that adhered between the rod and the holder was broken. It was then possible to bring the fiber through the surface without having it pulled onto the glass rod by a contracting film of water. The fiber but not the glass holder was dipped for less than 1 second in a wash solution consisting of 9 parts of isotonic mannitol, 0.24 M, and 1 part of isotonic sodium nitrate, 0.12 M. The wash solution used in this work was chosen to give the advantages of a non-electrolyte washing fluid, good mechanical removal of external fluid, and no opportunity for ion exchange, without producing the striking electrical effects caused by complete absence of electrolytes (4).

After the fiber was washed, the tendon attachments were blotted with filter paper and the fiber allowed to dry for about 10 seconds. The dry fiber was then laid across a depression in the waxed slide and cut 0.5 mm. from each end with a razor blade. Drops of silver nitrate and nitric acid were added and blown together over the fiber. It was found that drops of nitric acid ingest dry fibers with considerable force, so that it was often only necessary to free the ends of the fiber from the cut in the wax to get the fiber entirely inside the drop. When necessary the fiber was manipulated with very fine waxed glass needles.

Horsehairs were mounted with wax on the ends of glass rods. The diameters were measured with a micrometer and the lengths of exposed hair with a mm. scale. After soaking in Ringer's solution for 1 to 5 minutes, the hair was momentarily dipped in wash solution and then passed successively through the silver nitrate drop and 2 nitric acid drops. Controls carried out on hairs soaked in wash solution showed that this procedure did not affect the blank determination.

3 drops could conveniently be accommodated on a single slide and in practice the first and third of these were blanks, while the second contained the fiber. In this way errors due to unequal deliveries from the silver nitrate pipette could be detected. In general the blanks agreed to within one division of the burette.

Results

To control the method a number of fibers were treated with 4 per cent formaldehyde in Ringer's solution and analyzed for chloride. Whole muscle was also treated in formaldehyde and analyzed for chloride by the method of Keys (7). The fibers averaged 69 ± 9.2 micromoles per cc. (1 micromole $= 10^{-6}$ mole), while whole muscle averaged 70 ± 3.0 micromoles per gm. Formaldehyde combines with the free amino groups of proteins, liberating acidic groups from zwitter ion combination. The resulting increase in base-binding power presumably accounts for the low chloride concentration in the formaldehyde-treated muscle, since such fibers would otherwise be expected to contain about 91 micromoles per gm. of chloride.

The close agreement of the chloride concentration in single fibers and whole muscle is an indication that the micromethod is suitable for the purpose. It also shows fairly certainly that no chloride had been washed out of the fiber. If allowance is made for the tissue spaces in the whole muscle, the single fiber values should be lower by 7 to 17 micromoles per gm., depending on the tissue space assumed. So it is possible that some Ringer's solution adhered to the surface of these fibers in spite of the washing.

Normal fibers giving conducted responses were analyzed for chloride. The average chloride content of these fibers was 15.5 micromoles per cc. ± 35 per cent. The results were also calculated as the thickness of a surface film of Ringer's solution which would contain all the chloride. The average thickness of such a surface film was 3.2×10^{-3} mm. ± 19 per cent. The fact that the variation in the results is so much lower when the chloride is assigned to the surface is an indication that the chloride found was probably present as a surface film.

The results are shown graphically in Fig. 1 where the chloride concentration is plotted as abscissa and the diameter as ordinate. The line A-A is calculated for a 3×10^{-3} mm. thick film of Ringer's solution outside a chloride-free fiber. If the chloride

were initially at a uniform concentration of 30 micromoles per cc. and a uniform external layer 10^{-2} mm. thick had been washed free of chloride, the results would tend to fall along the line B-B. As is evident from Fig. 1, the results are more compatible with the hypothesis that the fiber is chloride-free but surrounded by a surface film containing 0.365×10^{-9} mole per sq. mm., corresponding to a film of Ringer's solution 3.2×10^{-3} mm. thick.
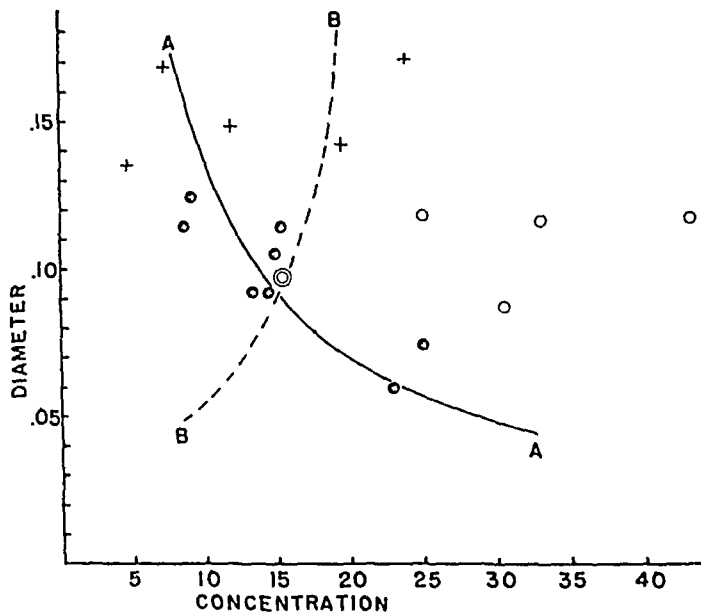
FIG. 1. Chloride in single muscle fibers. The concentration is expressed in micromoles per cc.; the diameter in mm. The solid circles represent normal conducting fibers; the double circle, the average concentration and diameter of the solid circles; the open circles, non-conducting fibers; the crosses, bullfrog fibers. See the text for an explanation of the curves A-A and B-B.

The experiments give no indication of where on the surface the chloride is located but it is logical to suppose that some of it is in the sarcolemma and the fragments of connective tissue that remain attached to the fiber after dissection.

In an effort to extend the range of diameters, fibers from jumbo Louisana bullfrogs, *Rana catesbiana*, were analyzed. There was unfortunately such a wide range of values that the results are nearly worthless. It is possible that the two high values were

caused by partial injury or poor condition of the fiber, as fibers which gave only local responses all showed higher chloride content than conducting fibers.

Horsehair with a surface film of Ringer's solution was washed in the same way as the fibers and analyzed for chloride on the surface. The mean thickness of the surface film was 0.92×10^{-3} mm. ± 24 per cent. The results are shown graphically in Fig. 2 where the apparent concentration of chloride in the hair is plotted as abscissa against the diameter as ordinate. The
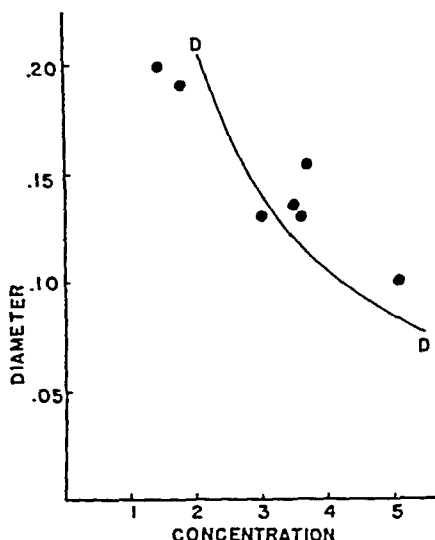
FIG. 2. Apparent concentration of chloride in horsehair. The units are the same as in Fig. 1.

curve D-D is calculated for a uniform surface film of Ringer's solution 0.92×10^{-3} mm. thick. The absolute value for the thickness of surface film that can withstand the washing technique is less than the apparent thickness found for fibers, but that may be partially explained by the fact that the horsehair was stiff and moved faster through the washing solution. It also seemed to have less Ringer's solution on its surface than the fibers. Of possible interest is the fact that the grouping of points around the line of equal thickness of film is similar in Figs. 1 and 2, the larger hairs having thinner films than the smaller ones.

DISCUSSION

Although single muscle fibers were found to contain chloride sufficient to produce an average concentration inside the fiber of 15 micromoles per cc., there is good evidence that most, if not all, of this chloride was on the surface of the fiber. The results showed a much lower probable error when calculated as the thickness of a surface film of Ringer's solution containing all the chloride than when calculated as a concentration of chloride inside the fiber. Another expression of the same fact is apparent in the way that the experimental points group themselves around a line calculated on the assumption that all the chloride is present as a uniform surface film of Ringer's solution. Horsehair models washed in the same way as the fiber showed surface films of Ringer's solution approximately one-third as thick as was calculated for the fibers but it seems likely that the horsehair was more efficiently washed because of its stiffness.

Since it has been shown that all of the chloride contained in muscle can be washed out by isotonic sugar solutions (1) or sodium sulfate (4), it would be difficult to prove that a longer period of washing was not removing chloride from inside the fiber. In these experiments the fact that the chloride appears to be on the surface of the fiber is indicative that none had been washed out of the inside.

It was impossible to blot the fibers thoroughly without causing injury which would be expected to make the fiber permeable to chloride, since fibers which gave only local responses had more chloride than fibers giving conducted responses. Both types of fibers had much less chloride than formaldehyde-treated fibers. It seems unlikely that isolated fibers could be completely free from chloride, since the sarcolemma and attached fragments of connective tissue probably contain chloride. The difficulty lies in the fact that the theoretical chloride-free fiber is not identical with the isolated fiber in its sarcolemma sheath.

Conway et al. (4) deduced a chloride concentration inside fibers in equilibrium with Ringer's solution of 21 micromoles per cc. which is clearly inconsistent with these results. They were probably measuring the difference between the tissue spaces and total chloride, so the presence of 16 per cent of injured fibers containing chloride would produce the same result as a high internal concentration of chloride. It is possible when muscle is soaked in Ring-

er's solution that some of the fibers lose their normal impermeability to chloride ions, thus accounting for the loss of potassium and equivalent gain of chloride which take place under these conditions.

Conway and Boyle (9) derived equations from which it may be deduced that in Ringer's solution the concentration of chloride inside a fiber permeable to chloride should equal the concentration of potassium in the Ringer's solution outside. The solution used in this work contained potassium at a concentration of 1.4 micromoles per cc. The hypothesis that the internal chloride concentration also equals 1.4 micromoles per cc. is just as consistent with these analyses as the hypothesis that the fiber contains no chloride. So, while this work does not confirm the work of Conway, Kane, Boyle, and O'Reilly (4), it is not at variance with the theoretical treatment of a chloride-permeable fiber outlined by Conway and Boyle (9).

SUMMARY

Isolated frog muscle fibers were analyzed for chloride. An average concentration of 15×10^{-6} mole per cc. of fiber was obtained but there were indications that most of this chloride was on the surface of the fiber. The results are not inconsistent with the assumption that the muscle fiber membrane is impermeable to chloride.

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AN APPARATUS FOR THE STUDY OF RAPID CHEMICAL REACTIONS*

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In 1923 Hartridge and Roughton (1) described an apparatus for the kinetic study of certain chemical reactions, which was based on the so called "flow principle." Later Millikan (2) joined Roughton to continue this work. Essentially, the apparatus of Roughton and his coworkers consists of a glass "observation tube" connected with a small "mixing chamber." The two solutions that are to react converge in the mixing chamber and then the mixture flows through the observation tube. If the reaction results in a change of color and if the flow along the tube is at a suitable and constant rate, the change of color occurs while the liquid is passing through the observation tube and the gradation of color along the tube remains fixed in position. If the rate of flow is known, the reaction time at any point along the tube may be calculated from its distance beyond the point of mixing. Colorimetric measurements by a photocell can be made at a number of points along the tube and a curve showing the kinetics of the reaction may be plotted. This procedure requires a fairly large amount of material and is therefore only satisfactory for substances such as hemoglobin that can be readily obtained in quantity. Furthermore, since each point on the curve requires a separate experiment, the method is somewhat laborious.

Since 1936 the writer and Dr. Kurt G. Stern have been studying certain rapid chemical reactions.

Various arrangements have been used in the course of this work; *e.g.*, a photoelectric method in which the solution of one

reactant was rapidly injected into the stationary solution of the second reactant (3) and a spectroscopic apparatus employing a falling plate camera for registration of the changes occurring during the reaction (4). The main reasons which led to the construction of the present machine were the desire to have the mixing more rapid and more reproducible and to study reactions of half times as short as 1 millisecond and as long as 100 milliseconds by an automatic, recording apparatus requiring only small quantities of material.

Thiel and Logemann (5) in 1934 described an apparatus of the flow type in which the observation tube was scanned during the experiment by a moving beam of light. By using a photocell and a recording galvanometer a record of the entire light absorption curve could be made in about the time required to get one point by the older method. This greatly reduced the total quantity of liquid that was required. In Thiel's apparatus, the lamp, observation tube, and photocell are all stationary, and moving prisms reflect the beam of light so that the light travels along the observation tube. The present author felt that while Thiel's idea should be followed in general it would simplify the construction to keep the light beam stationary and instead to move the observation tube vertically through the beam of light.

At Dr. Stern's suggestion and with his help the writer undertook the construction of the apparatus described in this paper.

Mechanical Construction

Fig. 1 shows diagrammatically the mechanical construction of the apparatus. It is mounted on a heavy stand with a flat wooden table top. Through a slot in the top there passes a frame constructed of steel shafting. This frame is guided by bearings and can slide vertically with respect to the table. Along the center line of the frame are two long machine screws which are turned by the operating mechanism. The top screw passes through a nut held stationary at the level of the table top and, when the screw is turned, the entire frame is raised or lowered depending upon the direction of rotation. The lower screw operates the two syringes which serve as the pumps of the machine. The observation tube is held by the frame just above the syringes and metal tubes connect the syringes with the bottom of the observa-

tion tube. The two screws are normally driven in opposite directions so that when the syringes are delivering liquids to the observation tube the tube is at the same time rising above the table. The storage vessels and the motor with its automatic limit switches and drive mechanism are not shown in Fig. 1.
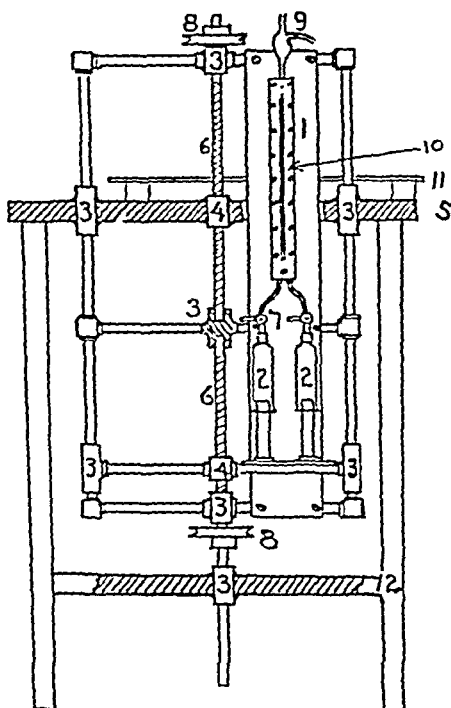
FIG. 1. Diagram to show the mechanical construction of the apparatus. *1* represents the observation tube, *2* syringes, *3* bearings, *4* nuts, *5* table top, *6* screws, *7* 3-way cocks, *8* pulley wheels, *9* glass liquid trap, *10* level of light beam, *11* calibration track, *12* lower table shelf.

The light path in Fig. 2 is above the table top and extends from the light source on one side of the observation tube to the photocell on the other. Monochromatic light must usually be used and though glass and gelatin color filters were tried, it was found to be more convenient to install a monochromator for this purpose.

There is a rotating sector in the light path to interrupt the beam

of light so that the output of the photocell may conveniently be amplified. This rotating sector is omitted if the light source is inherently pulsating, as for instance when an A.C. mercury arc lamp is used.

The photocell is connected with an amplifier and this is followed by a recording galvanometer. Thus changes in light absorption along the observation tube are recorded by the curve traced on the photosensitive paper of the galvanometer camera (Cardiette).

As the deflections of the galvanometer may not be proportional to the changes in light absorption, it is necessary to calibrate the
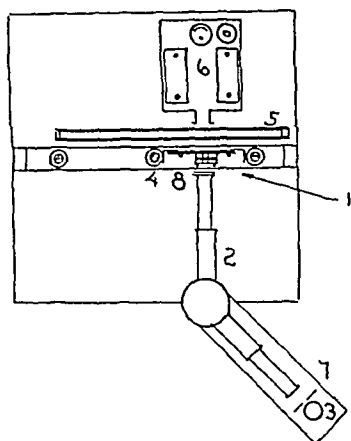
FIG. 2. Diagram of table top to show optical arrangement. *1* represents the observation tube, *2* monochromator, *3* light source, *4* nut for screw, *5* calibration track, *6* box containing photocell, *7* vertical slit, *8* horizontal slit.

apparatus. For this purpose an automatic calibrating mechanism is provided. Between the observation tube and the box containing the photocell there is a horizontal track which crosses the table top just beneath the path of the light beam and on this track runs a small carriage. This carriage holds two hollow glass wedges placed together to form a parallelogram. These wedges are filled with liquids which in light absorption are equivalent to the liquid in the observation tube before and after the reaction. When the calibration is to be made the observation tube is filled with water and then the carriage with the wedges is drawn across the light path by a speed-governed motor. The line traced by

the recording galvanometer in this case is the calibration curve; it is a straight line when the galvanometer deflection is inversely proportional to light absorption.

To evaluate the curves it is necessary to know the rate of flow through the observation tube and the rate at which the tube is scanned by the beam of light. It is convenient to have these rates automatically registered on the photographic paper simultaneously with the reaction curve. For this purpose two contact scales are provided, one of them to close an electric circuit at each cm. interval of syringe compression and the other to close an electric circuit at each cm. interval of observation tube movement. These electrical circuits control two vacuum tube relays which in turn control the flashing of two small neon-mercury sign lamps located in the recording galvanometer. Small lenses and mirrors focus the signals from these lamps on the edges of the photographic paper. The recording galvanometer is also provided with a time marker so that each curve taken is accompanied by a record of all the constants necessary for the calculation, provided, of course, that the observation tube and syringes have previously been calibrated.

The carriage which holds the calibration wedges is also provided with a contact scale, and when the calibration curve is traced fiducial marks on the photographic paper indicate the ratio of thickness of the two wedges at ten points along the calibration curve. From these data it is possible to express the galvanometer deflections, recorded during the main reaction, in terms of percentage of its completion.

Optical System

A movable bracket attached to the table holds the light source. This bracket is made in the form of an optical bench to hold any type of lamp desired. An 85 watt high pressure mercury vapor lamp (General Electric, type H-3) has been used during the hemoglobin experiments described below. A low voltage concentrated tungsten filament lamp (sound-track exciter lamp) has been used in other experiments.

The monochromator which is made from a simple spectrometer of the Bunsen-Kirchhoff type focuses a vertical slit located close to the light source as a monochromatic line upon the observation

tube. A second slit, this one horizontal, is located as close to the observation tube as possible. Thus the observation tube is crossed by a narrow, rectangular beam of light.

The photocell is placed behind the observation tube and at such a distance that the diverging beam of light illuminates the entire cathode. It is contained in a metal box, together with the first radio tube and the necessary batteries. At present a gas-filled cesium photocell (General Electric, type PJ-23) is being used. Although the output current of this tube is not strictly proportional to the intensity of the illumination, the fact that a calibration curve can always be made by the wedges permits its use.

Slight mechanical vibrations in the optical tract were found to give serious irregularities in the camera record but this difficulty was overcome by bracing the optical parts very rigidly.

Observation Tube

A cylindrical glass observation tube, as used by Millikan (6), was first tried but the reaction curves obtained by scanning were very irregular. Thiel used an observation tube made of two long and narrow glass plates with a slotted strip of metal clamped between them. His mixing chamber was entirely of metal and rather difficult to make. We found that by using plates of a transparent plastic, instead of glass, a very good observation tube can be made. If the plastic is unscratched, it is optically as good as plate glass. Furthermore, since the plastic can be drilled and cut, the design and construction of the mixing chamber are much simplified. The plastic we have been using is called Plexiglas and is made by the Röhm and Haas Company of Philadelphia. Two strips of this plastic of $10 \times 1 \times \frac{1}{4}$ inches with a slotted metal spacer between them, an inch wide and 10 inches long, form the tube. The thickness of the spacer and the width of the slot milled in it determine the cross-sectional area of the tube. There are also in front and back slotted cover plates of heavy brass connected by screws passing through holes drilled in the plastic side strips and metal spacer. The back plate is fixed in position in the frame of the machine. There is no trouble due to leakage when the screws are tight.

Undoubtedly the flow of liquid through a narrow tube of

rectangular cross-section is far from uniform. The rate will be relatively rapid at the center of the tube, slower along the sides, and still slower in the corners of the tube. There will, however, be a continuous interchange of liquid between these paths, and this turbulence will tend to prevent the liquid that has reacted to a certain extent from being spread out very far along the tube. Turbulence depends upon both velocity and geometrical shape, and a rectangular tube should show more turbulence than a circular tube. In any case, the optically determined reaction time represents an average value. The fast central column would actually reach the point corresponding to 50 per cent reaction further up the tube, and the slower columns not so far up. But the velocity value used in the calculations is also an average value, and so the errors involved tend to cancel each other out. Roughton and Millikan (7) in discussing this conclude that only negligible errors may be expected as due to this non-uniformity of flow.

Two tubes have been made so far, one with a cross-section of 0.05 cm. by 0.05 cm. and the other of 0.175 cm. by 0.1625 cm. The latter has been used in experiments with hemoglobin and erythrocruorin.

Mixing

As the plastic can be machined, it is possible to drill ducts through it to carry the reactants to the point where they meet. Ducts can also be made by grooving the inner surfaces of the plastic side strips or by engraving the sides of the metal spacer. There are many possibilities of design and a number of designs were tried.

To study the efficiency of mixing, three methods of observation were used. The first and simplest method was to use dilute ink in one syringe and water in the other, and to take photomicrographs of the mixing. This method, however, does not permit one to distinguish between fair mixing and complete homogeneity. The second method was to use a bentonite sol[1] in both syringes and to place Polaroid disks (set at 45° each side of the vertical) before and behind the observation tube, as sug-

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[1] The author wishes to thank Professor Hauser for this sol which contained particles of 80 to 200 mμ diameter.

gested by Hauser (8). With a low powered microscope the lines of flow due to the birefringence of the particles could be studied, but this method too does not show when mixing is complete. Finally, the well known Toepler schlieren method was used, the set-up consisting of a mercury lamp with a narrow horizontal slit followed by a large lens in front of the observation tube, and, then, at a distance beyond the tube, a horizontal adjustable knife edge and a telescope fitted with a camera. The large lens focused the image of the slit just above the knife edge and on the front lens of the telescope, while the telescope was focused on the observation tube. One syringe was filled with a 10 per cent solution of KCl and the other with water. When the liquids were flowing, the image of the lower part of the tube, where mixing was incomplete, appeared dark, but higher up, as mixing progressed, the image cleared, becoming bright when mixing was complete.

In general it was found that a few high velocity mixing jets, entering at the base of the tube, were preferable to a large number of jets of lower velocity. Thus poor results were obtained with an eight jet mixer, since in this case the jet velocity could not be made very much greater than the velocity of flow up the tube. One arrangement tried was to have two high velocity fan-shaped jets enter at the base of the flow tube, one from each side. This was done by scraping away the surface from a small area of the metal spacing strip on each side, just below the bottom of the central slot, and engraving ducts in the surfaces of the plastic side strips so as to bring the liquids to the scraped areas. The ducts could come to within a few mm. of the bottom of the observation tube and if the scraping were carefully done very high jet velocities were possible.

This mixing arrangement was later improved by the addition of a small "homogenizer" made on the colloid mill principle, and located at the bottom of the observation tube. To construct this, first a small slot crossing the bottom of the observation tube was filed in the metal spacing strip. Then two sections of small brass machine screws (2-56) cut to the length of the cross slot were pressed into that slot from either side so as to mesh in the center of the tube. These screws were soldered to the spacer at their ends to hold them in position and were then filed down to the

surface of the spacing strip. They were also filed from above to give a flat bottom to the observation tube. The result was that immediately after the initial mixing the reactants had to pass together through a 0.2 mm. zigzag slot, with great turbulence, appearing above the homogenizer ready for observation less than 0.0005 second after their entrance below.

When this arrangement was tested by the schlieren method no solid black phase could be seen, though there was a shading at the bottom of the tube to indicate that there some mixing was still in progress. This shading persisted for a distance of 3 mm. up the tube (corresponding to about 0.005 second), gradually disappearing. Undoubtedly, mixing was largely completed in the homogenizer.

A disadvantage of using synthetic plastics instead of glass is that the surface of the plastic is easily scratched. It cannot be rubbed without injury; even lens paper has to be used cautiously. It is perhaps better to clean it by soaking it in the solution of a detergent (e.g. Duponol).[2] Also its temperature coefficient of expansion is so different from brass that it should not be left with the screws tight except when the machine is in use. The photocell responds to imperfections hardly noticeable to the eye and if the plastic is scratched the record curve of the experiment is irregular.

Electrical Circuit

The electrical circuit is shown in the wiring diagram, Fig. 3. The first tube is a Western Electric No. 259B tube, chosen for its low grid current. At the voltages applied it operates under "floating grid" conditions which allow the use of a high resistance in the circuit of the photocell, giving high sensitivity. It should be noted that this tube is not connected as an amplifier but as an "impedance changer" and is therefore only slightly sensitive to vibration. A low impedance circuit leads from this tube to the amplifier proper which is best located at a distance away from all mechanical vibration. The tubes that follow are all of the type 6C6. The first set can be connected as pentodes if more amplification is desired. The last two are connected in parallel with the

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[2] Lately, carbon tetrachloride has been used for cleaning of the plastic strips with satisfactory results.

screen grids biased by a C battery. This C battery is adjusted so that when no light is falling on the photocell, the current output is only just above zero. Then when light impulses reach the photocell the galvanometer responds as it would to direct current.

Procedure

The procedure followed in testing is first to explore the reaction with the aid of small test cells, each having the same thickness as the observation tube, but holding only a few drops of liquid. A block of these cells is made by cutting notches in a strip of metal and by clamping sheets of plastic on each side. The metal
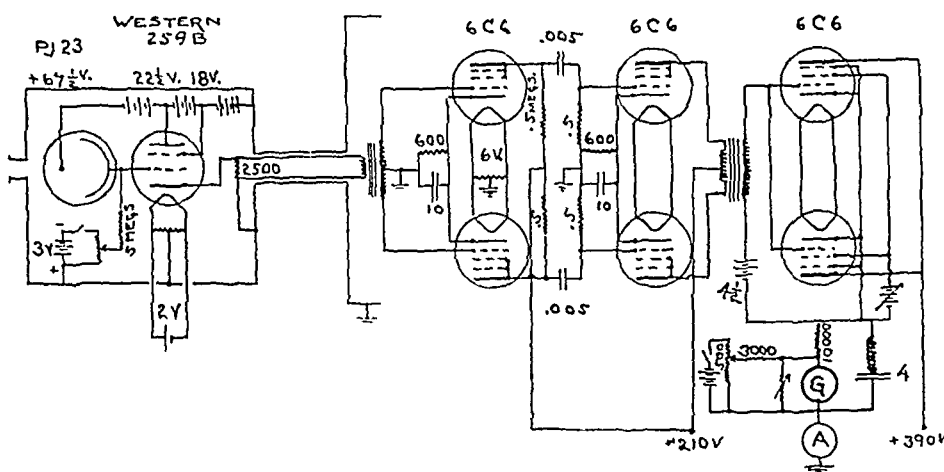
Fig. 3. Diagram of the electrical circuit

strip has the same thickness as the spacer used in the construction of the observation tube. This block of cells fits on the carriage that is used for the calibration wedges, and the cells can be brought into the beam of light one at a time. These cells are used to determine the best wave-length of light for the experiment and also the concentration of the reactants that will give satisfactory curves during the test runs. It is, of course, desirable to have a large deflection of the galvanometer but the deflection had best be kept within certain maximum and minimum limits where it is fairly proportional to the light absorption. In the experiments with hemoglobin, with the 436 mμ Hg line, almost perfect proportionality was found when a 0.1 per cent hemoglobin

solution (0.2 per cent in the syringe giving a 0.1 per cent solution in the observation tube) is being reduced. If, however, much more concentrated solutions were used, the light extinction was excessive before the reaction was complete.

Once the suitable concentration of the reactants has been determined, it is possible to calculate the concentration to be used in the calibration wedges, which will give the same galvanometer deflection as found for the test cells, and the calibration curve is then made (see Fig. 4).

Before the actual reaction curve is recorded, a control curve should be made with the syringes so filled that no reaction takes place in the observation tube. For instance, in the hemoglobin experiments one syringe was filled with 0.2 per cent hemoglobin and the other with water. This curve shows throughout the galvanometer deflection corresponding to the start of the reaction, modified by any irregularities that are due to the surface condition
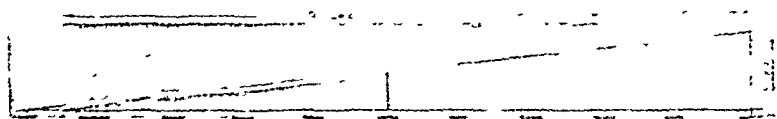
FIG. 4. Calibration curve, with straight lines superimposed

of the observation tube. These irregularities will, of course, be repeated in the reaction curve and, if necessary, the final curve may be corrected for them.

At the top of the observation tube there is a small glass trap that retains about 1 cc. of the liquid that has passed through the observation tube. After the reaction curve has been recorded, this liquid is sucked back into the tube, and a base-line corresponding to the level of 100 per cent completion of the reaction is added to the record by again starting the camera. Finally, the temperature and pH of the reaction mixture are measured.

Calculation of Results

For any syringes used, the delivery of liquid in cc. per cm. compression of the syringes must be measured. Let this be designated as V. For any observation tube used, the cross-section must be determined by a micrometer and measuring micro-

scope.   Let this be A.   The rate of flow along the tube is then $A/V \times S$ (seconds per cm.) where S is the time in seconds required for 1 cm. of syringe compression.   If the tube is rising at the rate of O seconds per cm. and if the record shows that after a certain time (R seconds) the reaction has progressed to a certain extent (usually to 50 per cent completion), then the distance along the observation tube from the point of mixing to that stage of the reaction will be R/O cm., and the time required by the reaction to that point will be $t = (A/V) \times (SR/O)$. A/V is a constant to which a correction must be applied if the time marker in the camera is "fast" or "slow."   The final form of the equation is $t = k (SR/O)$ seconds.

A sample reaction curve is shown in Fig. 5.   The value of S is given by the distance between the centers of the syringe signal
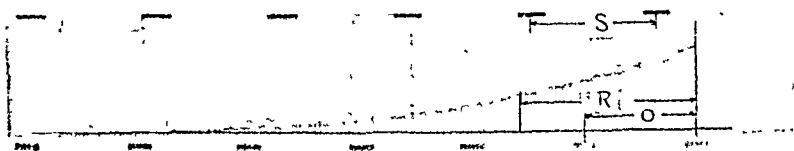
FIG. 5. A reaction curve with dimensional lines added for the purpose of calculation. S represents the time (by time scale) for 1 cm. of syringe compression, (O) the time for 1 cm. of observation tube movement, R the time for the observation tube to move through the light beam from the level of 50 per cent reaction to the level of the point of mixing.   The corresponding time of reaction is given by the equation $t_{50} = k(SR/O)$.

markings at the top of the record, expressed in seconds as measured by the time scale of the curve; and in the same way the bottom line of flow tube signal markings gives the value of O.

In the determination of R the calibration curve must be considered.   The ordinate of the reaction curve corresponding to say 50 per cent reaction is equal in height to the center ordinate of the calibration curve (corrected by proportion if the maximum deflections of the two curves are not the same) and the position of this ordinate of the reaction curve must be located.   The value R is measured horizontally from this ordinate to a vertical line drawn through the point on the curve corresponding to the point of mixing.   It is expressed in seconds according to the time scale of the curve.   The point of mixing is given as the center point of one of the lower line of signal marks.   The maximum deflection

D. DuBois

used in the calculation of R is slightly greater than the maximum deflection of the curve. It is found by extending the curve to meet the vertical line of the point of mixing. The reason for this discrepancy is that the beam of light has a vertical dimension and that, at the point of mixing, half of the light is already cut off by the base of the observation tube.

If the calibration curve is a straight line, the ordinate corresponding to 50 per cent reaction is equal to half the ordinate at the point of mixing.

TABLE I

Half Time Values (t_{50}) As Measured during Dissociation of Human Oxyhemoglobin

Temperature	pH	t_{50}	Temperature	pH	t_{50}
°C.		sec.	°C.		sec.
24		0.030	25	8.4	0.034
20	8.6	0.028	26	8.64	0.036
26.5	8.9	0.022	25.5	8.45	0.028
26.5	8.9	0.022	26	8.64	0.033
27	8.4	0.026	27	8.4	0.027
26	8.4	0.026	27.1	8.4	0.028
26	9.5	0.024	27	8.6	0.027
26	9.5	0.024	27	8.6	0.030
23	8.65	0.036			
Average.........					0.028

Tests on Hemoglobin

In order to check this apparatus as it was being developed, and also to obtain additional data, a series of determinations of the time required for the half reduction of human oxyhemoglobin was made. Millikan (9) gives this as 0.038 second at 22° and pH 8.6. He also shows that the rate is independent of pH for values above 8.1.

In these tests mixed blood samples from the New Haven Hospital were used. The corpuscles were washed with saline at the centrifuge, laked, and, after sedimentation of the ghosts at high speed, the hemoglobin was diluted to 0.2 per cent with a 0.2 M borate buffer of pH 8.6. The reducing agent was sodium hydrosulfite, 500 mg. of which were dissolved in 100 cc. of 0.2 M boiled

borate buffer, adjusted to give a pH of approximately 8.6 to the reaction product.

Disregarding the earliest experiments which were inaccurate due to the method used in taking a base-line, we give results obtained since October, 1939 (Table I).

These results are somewhat lower than Millikan's t_{50} value of 0.038 second but the temperatures were higher, which might account for some of the discrepancy. However, considering the differences in construction of the two machines, the agreement appears rather satisfactory.

SUMMARY

An apparatus has been built for the study of the kinetics of certain rapid chemical reactions. This apparatus is a modification of that described by Thiel and Logemann and is based on the Hartridge-Roughton flow principle. The machine records automatically, in the form of a curve on a time scale, the changes in light absorption that occur during the reaction.

The novel features of the apparatus are:

1. The observation tube moves vertically through a fixed beam of monochromatic light so that the light scans the tube during the reaction.

2. The observation tube is formed with bars of transparent plastic, and the ducts which carry the reactants to the point at the base of the observation tube, where they mix, are cut or drilled in this plastic material.

3. The process of mixing has been studied by various optical methods including the schlieren method, and from a number of mixing arrangements tried one has been selected which gives satisfactory mixing. This mixer is built on the homogenizer or colloid mill principle.

4. A double wedge calibrating device is used to trace a calibration curve of the apparatus as a whole.

5. As the apparatus functions, small lights flash and make fiducial marks on the edges of the photographic record. These marks together with the reaction curve complete the data necessary for a calculation of the kinetics of the reaction under study.

Tests made on the reduction time of oxyhemoglobin are compared with previous results reported by Millikan.

Addendum—While the present paper was being prepared for publication, Chance described an ingenious, recording apparatus (10) employing the "accelerated" and the "stopped" flow principles for the study of rapid reactions. The time range covered by the apparatus of Dr. Chance (0.3 millisecond to 60 seconds) and its fluid economy are similar to those characteristic of the arrangement here described.

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ANTICOMPLEMENTARY FACTOR IN FRESH YEAST*

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Although the inactivation of complement by yeast cells was
described by von Dungern (1) as early as 1900, the nature of the
phenomenon has remained obscure.

It has been established by Coca (2) that the inactivation by
yeast is due to the removal or destruction of a relatively heat-
resistant fraction of complement, and not to the introduction of
inhibiting substances. Furthermore, Whitehead, Gordon, and
Wormall (3) employed a zymin preparation, and found that it
combined either physically or chemically with the third component
of complement.

The present report deals with the preparation of an insoluble
fraction from fresh yeast which can inactivate, specifically, the
third component of complement.

Materials and Methods

The method of McAnally and Smedley-MacLean (4) was used
to fractionate the yeast, but the employment of 40 per cent KOH,
as called for in this method, appeared to be too drastic, inasmuch
as the resultant insoluble fraction had little or no ability to inac-
tivate the third component of complement. The method em-
ployed for the preparation of the insoluble, active fraction follows.

500 gm. of fresh Fleischmann's yeast were suspended in 2
liters of 0.5 M Na_2HPO_4, boiled for 1 hour, and then cooled. 0.5
gm. of purified trypsin (Wilson special trypsin) was added together
with a few drops of toluene, and the mixture was incubated at
37° for 16 days. At the end of this time the mixture was negative

to protein tests. The supernatant fluid was decanted and discarded. 2 volumes of 95 per cent ethyl alcohol were added to the residue; the latter was then filtered and washed six times with 95 per cent alcohol. The supernatants and washings were discarded. The precipitate (Fraction A) was added to 4 liters of warm water, boiled for 30 minutes, and allowed to settle; the supernatant was syphoned off. This process was repeated two times. The original precipitate (Fraction A) was boiled several times with distilled water and centrifuged each time while hot, the process being repeated until the acid hydrolysis products of the supernatant liquids failed to reduce Fehling's solution. The residue was washed repeatedly with absolute alcohol and dried as completely as possible. The dried material was then refluxed for 3 hours with absolute alcohol, filtered, and finally dried *in vacuo*. The resultant product was a fine, almost white, hygroscopic powder, which was insoluble in hot water, organic solvents, and cold alkali. The yield was about 2 per cent. Chemical analysis revealed carbohydrate 94 per cent, nitrogen 1.78, magnesium 2.43, phosphorus 0.4. This fraction was labeled the "insoluble fraction," and it proved to be the agent responsible for the inactivation of the third component.

This material was further fractionated by the following procedure.

2 gm. of the insoluble fraction were added to 100 cc. of cold 1 N HCl, and the mixture was kept overnight in the refrigerator at a temperature of about 3°. The suspension was then centrifuged, and the supernatant, which contained the magnesium-phosphorus complex (4) was decanted. The precipitate (Fraction B) was added to 1 liter of warm water, boiled for 1 hour, and centrifuged while hot. The undissolved material was boiled again several times with distilled water, and processed by a method similar to that used in the preparation of the original insoluble fraction. The product was designated as Fraction C, and contained 0.54 per cent nitrogen. All of the insoluble fractions were stored *in vacuo*, since, upon long exposure to air, they adsorbed moisture and changes occurred so that they no longer inactivated the third component. When such changes occurred, the original activity was restored by refluxing the material again with absolute alcohol.

Immunological

The methods employed in this laboratory for complement titrations and for reactivation procedures have been previously described (5). The following method was used in testing the fractions.

Various amounts of the insoluble fractions were added to 50 volumes of distilled water, boiled for 30 minutes, and centrifuged. The supernatants were drained, and the residues allowed to cool. 1 cc. of fresh guinea pig serum was added to each fraction, thoroughly mixed with the fraction, and the suspensions were placed in a water bath at 37°. The mixtures were shaken by hand every 10 minutes. After incubation for 2 hours, the mixtures were made up to 10 cc. with 0.9 per cent NaCl solution and centrifuged

TABLE I

Action of Various Constituents of Yeast on Third Component of Complement

Yeast fraction	Minimal amount necessary for inactivation of third component of complement
	mg.
Fraction A	250
" B	50
" C	50
Insoluble fraction	10
Fresh yeast	250

clear. The pH of the supernatants was adjusted to 7.2, and complementary activities were immediately determined. Reactivation experiments were carried out directly afterward. The soluble fractions were added directly to 1 cc. of serum, and treated thereafter in the same manner as were the insoluble fractions. The amounts employed and the anticomplementary action of each fraction are given in Table I.

Results

It is seen in Table I that the insoluble fraction inactivated the third component of complement in amounts only one-twenty-fifth of the required amount of fresh yeast. After the cold HCl treatment of the insoluble fraction, which resulted in a loss of 25 per

cent of the carbohydrate, a loss of 70 per cent of the total nitrogen, and a loss of 75 per cent of the magnesium-phosphorus complex, the insoluble fraction still possessed marked anticomplementary power. *None of the soluble fractions inactivated the third component.* Since all of the active, isolated fractions were insoluble, it is altogether possible that the inactivation of the third component is due to an adsorption of a heat-resistant component of blood serum. Further study of the phenomenon is in progress.

SUMMARY

An insoluble fraction isolated from fresh Fleischmann's yeast is the agent responsible for the anticomplementary properties of the yeast.

It is suggested that the inactivation of the third component of complement is due to the adsorption of a relatively heat-resistant fraction of blood serum.

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OSMOTIC PRESSURE OF EGG ALBUMIN SOLUTIONS

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Values for the molecular weight of egg albumin have been reported on numerous occasions. There is, however, a disconcerting lack of agreement between these values as is shown in Table I.

It is the purpose of this paper to attempt to eliminate some of the uncertainty regarding the molecular weight of egg albumin, with a new and simple osmotic pressure apparatus. Some related considerations are reported.

EXPERIMENTAL

The form of the osmotic pressure apparatus finally adopted was inspired by that of Oakley (12). Its construction, shown in Fig. 1, is largely self-explanatory. All glass parts were made of Pyrex. The capillary tube which was 0.2 mm. in diameter was furnished by the Corning Glass Works. Its uniformity of bore was tested and found to be sufficient for our purposes. The collodion sac which contains the buffered protein solution is attached to the indicated glass tube by first coating the glass tube with stop-cock grease and then slipping the sac over the end of the tube and winding tightly with rubber bands. The 2-hole rubber stopper, with the collodion sac filled with buffered protein solution (about 20 cc. are required for each experiment) in place, is coated with stop-cock grease and inserted into the larger tube which is completely filled with buffer. The rubber stopper is held in place by means of a metal clamp (not shown in Fig. 1). Toluene is added from the top of the capillary and forced down the capillary with a gentle air pressure. Small, 1-hole, rubber stoppers are placed in the tubes containing the buffer solution and the protein

solution.   Their purpose is to decrease evaporation during the experiment.

The whole apparatus is clamped in a fixed position in a constant temperature bath at 25°.   The stop-cock is allowed to remain open and the levels of the menisci of the buffer solution, of the protein solution, and of the toluene observed with a cathetometer.

TABLE I

Values for Molecular Weight of Egg Albumin Reported by Various Workers

Method	Mol. wt.	Workers	Bibliographic reference No
Osmotic pressure .	34,000	Sørensen	1
Chemical analysis	33,800	Cohn, Hendry, Prentiss	2
Ultracentrifuge (equilibrium)	34,500	Svedberg and Nichols	3
Recalculation of Sørensen's data	43,000	Adair	4
Osmotic pressure ..	43,000	Marrack and Hewitt	5
" " (1 determination)	36,000	Burk and Greenberg	6
" "	46,000	Taylor, Adair, Adair	7
Chemical analysis	35,700	Bergmann and Niemann	8
Ultracentrifuge (equilibrium)	40,500	Svedberg and Pedersen	9
" (sedimentation)	44,000	Svedberg and Pedersen	9*
Chemical analysis	36,800	Bernhart	11
Osmotic pressure .	45,160	Bull (present investigation)	

* If the diffusion constant of Polson (10) is used, a molecular weight of 44,500 is obtained.

The stop-cock is then closed.   The toluene meniscus immediately begins to drop as buffer solution flows into the collodion sac. Equilibrium is usually attained within 2 hours, although observations are continued for several hours thereafter to be certain that equilibrium has been reached (usually overnight).   At the end of the experiment the stop-cock is opened and the initial levels of the buffer solution, of the protein solution, and of the toluene are

checked. The difference in the level between the protein solution and the buffer solution is converted into cm. of water. Likewise the difference in the level between the initial and equilibrium positions of the toluene meniscus is converted into cm. of water. The osmotic pressure is calculated as the sum of these two pressures.

This method of calculation introduces two small errors. During a measurement the toluene-buffer meniscus also drops, owing to flow of buffer into the collodion sac. This means that the change
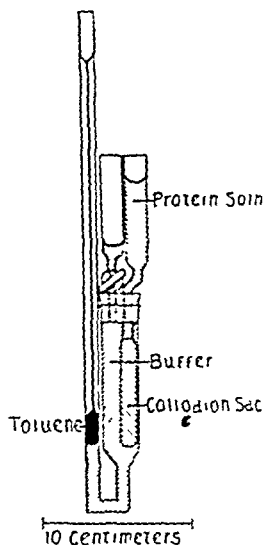
FIG. 1. Osmotic pressure apparatus

in hydrostatic pressure will not be exactly given by multiplying the change in height of the toluene-air meniscus by the specific gravity of toluene. The change in level of the toluene-buffer meniscus is so extremely small, however, owing to the size of the capillary, that it has been neglected. Another error which is somewhat ambiguous arises from the fact that the specific gravities of the buffer solution and the protein solution are not exactly equal, which means that the hydrostatic pressure difference between the outside and inside of the collodion sac is not everywhere the same and is not exactly equal to the difference in level

between the buffer and protein solution menisci multiplied by the specific gravity of the protein solution. Calculation shows that this error amounts to less than 1 per cent even in the more concentrated protein solutions. Neglecting this correction tends to give a lower osmotic pressure than is actually the case and accordingly a smaller molecular weight would be obtained if the correction were applied. Actually, this effect is almost exactly counterbalanced by a neglect of protein hydration to be discussed later on.

The apparatus is easily and quickly constructed at a very modest cost. The only part of it which requires some skill and practice to make is the collodion sacs. These should be uniform and of the proper porosity. The method used is to fill a clean test-tube of the proper dimension with Merck's u.s.p. collodion. The tube is then emptied as completely as possible of collodion, but not allowed to drain. The tube is then rotated slowly in a horizontal position until the collodion is dried sufficiently so that it does not flow. The tube is then connected to a vacuum line and dried for 15 minutes. The tube receives another treatment with collodion and is again dried. The sac is then removed from the test-tube with the aid of water.

During an experiment, the temperature of the water bath fluctuated between certain narrow limits. This fluctuation in temperature produced expansions and contractions of the fluid within the osmotic pressure apparatus which were reflected in movements of the toluene meniscus. These movements of the toluene meniscus amounted in some cases to as much as 2 mm. In order to measure the osmotic pressure, it was necessary to plot the position of the toluene meniscus as a function of time at minute intervals for 10 minutes, and then draw a median line through the distorted sine wave curve. The median line was taken to represent the true pressure.

The egg albumin was prepared by the method of Kekwick and Cannan (13) and recrystallized three times. It was then dialyzed against distilled water until it was sulfate-free. The protein concentration was determined by drying a given volume of the solution to a constant weight at 105° in a vacuum oven. Three preparations of egg albumin were used in this investigation; the results from the three preparations were indistinguishable.

Results

Fig. 2 shows the course of attainment of osmotic equilibrium across an unusually dense collodion sac (required a longer time than usual to reach equilibrium). The lower curve gives the results for equal concentrations of buffer inside and outside of the collodion sac, while the upper curve illustrates what happens when the concentration of the buffer inside (0.1 M) is twice as great as that outside (0.05 M). Evidently the same osmotic pressure is attained irrespective of initial distribution of buffer and accordingly small errors in buffer concentrations will not affect the results.
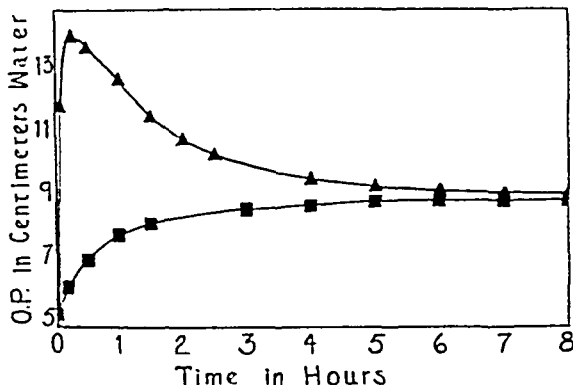
FIG. 2. Registered osmotic pressure as a function of time. The lower curve represents 0.05 N sodium acetate buffer inside and outside the collodion sac; the upper curve, 0.05 N sodium acetate buffer outside and 0.10 N inside.

Table II shows the osmotic pressure data of egg albumin solutions in a 0.05 M sodium acetate buffer at pH 4.70. The molecular weights have been calculated by use of the equation of Burk and Greenberg (6), $M = CdRT/100P$, where C is the protein concentration in gm. per 100 gm. of buffer solution, d is the density of the buffer solution, R is the gas constant and is expressed in cc.-cm. of water per degree, T is the absolute temperature, and P is the osmotic pressure in cm. of water (at 25°). Inserting numerical values for the constants, we have $M = 2.530 \times 10^5 \times C/P$.

In the calculation of the molecular weight of egg albumin the

first two values in Table II were excluded because it was felt that the osmotic pressures were so small in these two cases that these determinations were not very accurate. The average of the remaining twenty-one values was 45,160 with a standard deviation of the mean of 180.

TABLE II

Osmotic Pressure of Egg Albumin Solutions

Protein concentration per 100 gm. buffer	Water pressure (25°)	$\frac{C}{P}$	Mol. wt.
gm.	*cm.*		
0.085	0.540	0.1574	39,822
0.257	1.289	0.1994	50,448
0.342	1.981	0.1726	43,668
0.428	2.441	0.1753	44,351
0.514	2.874	0.1788	45,236
0.687	3.837	0.1790	45,287
0.859	5.020	0.1711	43,288
1.032	5.917	0.1744	44,123
1.292	6.981	0.1851	46,830
1.553	8.772	0.1770	44,781
1.729	9.405	0.1838	46,501
1.948	10.980	0.1774	44,882
2.080	11.609	0.1792	45,338
2.168	12.203	0.1777	44,958
2.484	13.933	0.1783	45,110
2.610	14.547	0.1794	45,388
2.610	14.560	0.1793	45,363
2.906	16.146	0.1800	45,540
3.230	17.889	0.1806	45,692
3.230	17.904	0.1802	45,601
3.230	17.749	0.1818	45,995
3.502	19.603	0.1786	45,186
3.759	21.019	0.1788	45,236

DISCUSSION

The molecular weight of 45,160 for egg albumin is in keeping with the osmotic pressure measurements of Adair *et al.* It is somewhat higher than that obtained by the ultracentrifuge and is not in agreement with the result from chemical analysis as calculated by Bergmann and Niemann (8).

It is to be emphasized that molecular weights calculated from osmotic pressure as reported in this paper are anhydrous molecular weights. What an osmotic pressure measurement does in essence is to indicate the number of particles which cannot pass through a membrane in a given weight of solution. The concentration of protein in solution is expressed on a dry weight basis and accordingly the molecular weight calculated on this basis gives a dry molecular weight. There is a small error introduced by the neglect of hydration. For example, egg albumin in solution is hydrated to the extent of about 36 per cent (14). This means that for a solution containing 2 gm. of egg albumin in 100 gm. of buffer solution 0.72 gm. of water is bound and presumably not available to act as solvent for the protein. The concentration of the protein is then effectively 100.72 per cent of the calculated value and accordingly the molecular weight would be greater if this correction were applied. As indicated previously, this error is in the opposite direction to and of the same order of magnitude as that introduced by the neglect of the difference in density of the protein solution and of the buffer solution. Both corrections have been neglected.

Both hydration and molecular asymmetry tend to increase the viscosity of protein solutions and to decrease the rate of diffusion of proteins in solution. It has not been possible to separate the influence of these two factors, and accordingly their true rôle in diffusion and in viscosity remains ambiguous. It is interesting to consider the case of egg albumin in light of the present research together with work reported in the literature.

Since, as indicated, egg albumin is probably hydrated to the extent of 36 per cent, its hydrated molecular weight must be 136 per cent of its anhydrous molecular weight. The anhydrous molecular weight is, as we have shown in the present investigation, 45,160 and accordingly the hydrated molecular weight of egg albumin is 61,438. The density of egg albumin containing 36 per cent water is 1.202 (14), and, therefore, the hydrated molecular volume of egg albumin is 51,113. If the egg albumin molecules be assumed to be spherical, the radius of the molecule is calculated to be 2.72×10^{-7} cm. Substituting this value in the Sutherland-Einstein diffusion equation for spherical particles, we find the diffusion constant of hydrated, spherical egg albumin molecules

in pure water and at 20° to be 7.79×10^{-7} sq. cm. per second. By the same method of calculation the diffusion constant of unhydrated, spherical egg albumin molecules (molecular weight of 45,160 and density of 1.34) in pure water and at 20° is 8.96×10^{-7} sq. cm. per second. The experimental value for the diffusion constant at 20° and in pure water is 7.76×10^{-7} sq. cm. per second (10). The agreement between the diffusion constant calculated for hydrated, spherical molecules and the experimental value is indeed remarkable and indicates that the egg albumin molecule is substantially spherical. It will be recalled that the above argument is practically identical with that of Adair and Adair (15). This type of calculation should be applied to other proteins in addition to egg albumin.

If 124 is granted as the average residue weight of egg albumin (8) and the carbohydrate content (16) is subtracted from the weight of the protein, the total number of residues in the egg albumin molecule is 354. This contrasts sharply with the value of 288 residues calculated by Bergmann and Niemann (8).

SUMMARY

1. An apparatus is described for the rapid and accurate determination of the osmotic pressure of protein solutions.

2. The molecular weight of egg albumin has been determined by osmotic pressure measurements and found to be 45,160. It is emphasized that this is an anhydrous molecular weight.

3. The hydrated molecular weight of egg albumin has been estimated to be 61,440. The diffusion constant of a spherical particle with this molecular weight has been calculated and compared with the experimental value. The close agreement of the two values is taken to indicate that the egg albumin molecule is essentially spherical.

4. The number of amino acid residues in egg albumin is calculated to be 354.

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STUDIES ON THE METHANE FERMENTATION

V. BIOCHEMICAL ACTIVITIES OF METHANOBACTERIUM OMELIANSKII

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The general equation approximately describing the reactions by which methane-producing bacteria obtain their energy is

$$4H_2A + CO_2 \rightarrow 4A + CH_4 + 2H_2O \qquad (1)$$
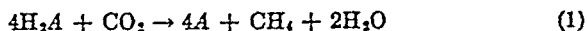
where H_2A represents an oxidizable compound, and A its oxidation product (1, 2). In this paper we shall consider principally three questions. (*a*) What oxidizable compounds can be used by *Methanobacterium omelianskii* and what transformations do they undergo? (*b*) What substances, if any, can replace CO_2 as an oxidizing agent? (*c*) How much of the substrate carbon is converted into cell material and what is its source, CO_2 or H_2A?

Oxidation Reactions

Qualitative Experiments—The ability of *Methanobacterium omelianskii* to oxidize various organic compounds has been determined by growth experiments with the following medium (Medium B) made up with tap water, oxidizable compound 0.3 to 1.0 per cent, K_2HPO_4 0.5, KH_2PO_4 0.7, $(NH_4)_2SO_4$ 0.03, $MgSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.001, and 2 per cent by volume of a saturated $CaSO_4$ solution. Immediately after autoclaving 1.0 to 1.5 cc. of a 1 per cent $NaS \cdot 9H_2O$ solution and 5 to 8 cc. of a 5 per cent Na_2CO_3 solution are added per 100 cc. The final pH is 7.4 to 7.7. All cultures were incubated under strictly anaerobic conditions at 35–38°. The details of the cultural technique and a description of the pure culture used are given elsewhere (3).

The experiments show that the only types of compounds utilized by *Methanobacterium omelianskii* are simple primary and secondary alcohols. Ethanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol, secondary butanol, and *n*-pentanol are oxidized. It is probable that higher alcohols of the same types can also be oxidized, though they have not been tested. Methanol and tertiary butanol are not attacked, nor are any of the other common organic substrates tested. These include formate, acetate, propionate, *n*-butyrate, *n*-valerate, lactate, malate, succinate, glucose, glycerol, mannitol, 2,3-butylene glycol, yeast autolysate, and tryptone. It is particularly noteworthy that neither the fatty acids nor glucose is decomposed.

It should be mentioned here that although media containing formic acid as the sole oxidizable constituent cannot support the growth of *Methanobacterium omelianskii*, formic acid can under certain conditions be decomposed. The decomposition of formic acid will be discussed further below.

The products of the oxidation of primary alcohols are the corresponding fatty acids. Acetic acid formed by the oxidation of ethanol was identified by the sodium uranyl acetate microchemical reaction and was shown to be pure acetic acid by Duclaux distillation. The other volatile acids (propionic, *n*-butyric, isobutyric, and valeric) were both identified and shown to be 95 to 100 per cent pure by the Duclaux method.

The secondary alcohols, isopropanol and isobutanol, are oxidized to the corresponding ketones, acetone and ethyl methyl ketone. These ketones were identified by the melting points of their 2,4-dinitrophenylhydrazones.

The quantities of the various oxidation products were in all instances fairly large. Although in this particular set of experiments accurate determinations of the quantities of alcohol oxidized and of products formed were not made, the two were undoubtedly of the same order of magnitude.

Oxidation of Ethanol. Quantitative Experiments—It has been shown above that acetic acid is the only volatile acid formed from ethanol. This does not exclude the possibility that other products such as carbon dioxide are also formed. Although a complete oxidation of ethanol appeared improbable in view of experiments previously carried out with enrichment cultures (1), still

it seemed highly desirable to check the point experimentally with a pure culture. Quantitative carbon balance experiments were therefore undertaken.

The fermentations were carried out in all-glass vessels containing about 250 cc. of Medium B. Incubation was at 37°; oxygen was removed by oxsorbent, an acid chromous chloride preparation. The evolved gases were collected over mercury. Samples of the medium were removed for analysis immediately after inoculation and after the fermentation had ceased (5 to 10 days). Ethanol was separated by distillation and estimated by Northrop's method (4). Acetic acid was determined by direct steam distillation and titration after suitable corrections for carbon dioxide and incomplete recovery were applied. Dissolved carbon dioxide was estimated by the Van Slyke manometric technique (5); gaseous carbon dioxide and methane were collected over mercury and were determined by ordinary methods of gas analysis. Dissolved methane was calculated from solubility data.

The results of four such experiments are presented in Table I. The experiments differ with respect to the substance limiting the catabolic reaction. In Experiments 1, 2, and 3, ethanol was present in excess; the quantity of carbon dioxide therefore determined the amount of alcohol oxidized. In Experiment 4 carbon dioxide was present in excess and ethanol was limiting.

Experiments 1, 2, and 3 of Table I show that acetic acid accounts for 93 to 96 per cent of the ethanol oxidized. The discrepancy between the ethanol oxidized and acetic acid formed cannot be due to a production of carbon dioxide, since if this occurred the net disappearance of carbon dioxide would be decreased. No such effect is evident; the uptake of carbon dioxide is if anything slightly greater than is required by Equation 2. It will be shown below that the discrepancy between ethanol and acetic acid is caused by conversion of part of the alcohol into cell material.

Since in Experiments 1, 2, and 3 the oxidizing agent, carbon dioxide, was limiting while ethanol was present in excess, it is not surprizing that the oxidation of the latter compound was incomplete. A much more rigorous test of the ability of *Methanobacterium omelianskii* to oxidize ethanol to carbon dioxide is provided by Experiment 4 in which carbon dioxide was present in excess while ethanol was limiting. Even under these conditions, how-

TABLE I

Oxidation of Ethanol

The data are expressed in мм. Columns A give observed quantities; Columns B give мм per 100 мм of ethanol decomposed.

Compound	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Theoretical*
	A	B	A	B	A	B	A	B	B
Ethanol	−17.0	−100	−11.8	−100	−16.6	−100	−10.0	−100	−100
Acetic acid	15.8	93.1	11.4	96.1	15.6	93.9	9.90	99.0	100
CO_2	−8.39	−49.5	−6.12	−51.7	−8.45	−50.9	−5.34	−53.4	−50
Methane	8.34	49.2	6.03	51.2	7.75	46.7	4.80	48.0	50

* According to Equation 2.

ever, the organism appears to be unable to oxidize ethanol beyond the stage of acetic acid.

From the above experiments it may be concluded that the overall catabolic reaction carried out by *Methanobacterium omelianskii* is closely described by the equation,

$$2C_2H_5OH + CO_2 \rightarrow 2CH_3COOH + CH_4 \qquad (2)$$

This is in agreement with the conclusion reached previously (1) from experiments with enrichment cultures.

Oxidation of Butanol—In order to see whether the oxidation of butanol goes beyond the stage of butyric acid, a quantitative fermentation experiment was undertaken in which butanol was limiting, carbon dioxide being present in excess. The medium and conditions were essentially the same as in the experiments described above except that butanol (0.5 volume per cent) was substituted for ethanol. The fermentation of butanol being much slower than the fermentation of ethanol, the culture (1000 cc.) was allowed to incubate for 21 days at 37°. At the end of this period gas production had practically ceased.

The analytical data showed that butyric acid (17.0 mM) accounts for 97 per cent of the butanol decomposed (17.5 mM). It may be concluded that the oxidation of butyric acid to 2 molecules of acetic acid, previously thought on the basis of experiments with enrichment cultures to be brought about by *Methanobacterium omelianskii* (1), is probably due to another organism.

Quantitative fermentation experiments with other alcohols have not been undertaken. It may be expected, however, on the basis of the qualitative results reported above that higher alcohols are in general oxidized (without regard to synthetic reactions) only as far as the corresponding fatty acids or ketones.

Reduction Reactions

The second main problem undertaken was to find out what compounds, if any, can replace carbon dioxide as an oxidizing agent for *Methanobacterium omelianskii*. The compounds that have been tested are methyl alcohol, formic acid, nitrate, sulfate, and oxygen.

A qualitative experiment to test the reducibility of the above compounds under normal physiological conditions was carried

out with growing cultures.   To Medium B containing an excess of ethanol as the oxidizable constituent, a small, limiting amount of carbon dioxide and 0.3 per cent of the various compounds to be tested (except oxygen) were added.   Oxygen was supplied by exposing the cultures (test-tubes) to air; all other cultures were incubated under strictly anaerobic conditions.   When all activity had ceased, the media were analyzed for acetic acid.   It was expected that any substance acting as an oxidizing agent would increase the yield of acetic acid over that amount equivalent to the carbon dioxide available.

The results showed that oxygen and nitrate are toxic, since no acid production occurred in their presence.   Sulfate and methanol appear to be completely inert, since they did not significantly increase or decrease the formation of acetic acid as compared with the control.   Formate is the only substance utilized.   Not only was the amount of acetic acid approximately doubled in the formate cultures but formate had almost completely disappeared.

Decomposition of Formic Acid—The decomposition of formic acid in the above experiment may appear to contradict earlier results (see above) which indicated that formic acid is not attacked.   The contradiction, however, is only apparent, for the conditions of the two experiments were quite different.   In the former formic acid was the sole oxidizable constituent of the medium, while in the latter ethanol was present as well.

In order further to clarify the conditions necessary for the decomposition of formic acid, the growth experiment summarized in Table II was set up.   The essential features of the media used are given in Columns 2, 3, and 4.   Column 5 shows whether or not growth occurred during 20 days incubation.   The remaining columns are self-explanatory.   The data given are averages of duplicate experiments.

The data show that *Methanobacterium omelianskii* cannot live with formic acid alone (Culture 1) or with formic acid plus carbon dioxide (Culture 2).   In the absence of ethanol no growth occurred.   These results corroborate the conclusion reached previously that formic acid cannot replace ethanol as the oxidizable substrate.   Cultures 4, 5, and 7, all of which contain ethanol, carbonate, and formate, show, however, that formic acid can be decomposed.   In all three cultures considerable quantities of

formic acid disappeared and there was a corresponding increase in acetic acid as compared to the control culture with alcohol and carbonate only (Culture 3). It is significant that the decomposition of formic acid is dependent upon the presence of carbon dioxide as well as of alcohol. Culture 6 shows that in the absence of carbon dioxide no growth and no significant formate decomposition occur.

We may conclude that formic acid, though decomposed under suitable conditions, cannot replace either carbon dioxide as a sole hydrogen acceptor or ethanol as a sole hydrogen donor in the

TABLE II

Conditions for Decomposition of Formic Acid

The quantities are expressed in mg. per 100 cc.

Culture No. (1)	Composition of medium			Growth (5)	Final HCOOH (6)	Decomposed HCOOH (7)	Acetic acid (8)
	C₂H₅OH (2)	HCOOH (3)	Na₂CO₃ (4)				
1	0	207	0*	−	207†	0†	0
2	0	207	100‡	−	207†	0†	0
3	700	0	100‡	+	0	0	237
4	700	67	100‡	+	17	50	311
5	700	138	100‡	+	36	102	355
6	700	207	0*	−	206	1	0
7	700	260	100‡	+	5	255	563

* CO_2 was removed with KOH.

† Estimated, not determined.

‡ Additional CO_2 was available from the K_2CO_3-pyrogallol mixture used for removing oxygen.

metabolism of *Methanobacterium omelianskii*. The failure to replace carbon dioxide strongly indicates that formic acid cannot be an actual intermediate in the reduction of carbon dioxide to methane. Nevertheless formic acid is ultimately reduced to methane, approximately 1 molecule of methane being formed per molecule of formate decomposed. In one experiment 11.1 mM of CH_4 were formed from 9.5 mM of CO_2 and 2.2 mM of HCOOH.

Further evidence that formic acid is not an intermediate in carbon dioxide reduction is furnished by measurements of the relative rates of formic acid and carbon dioxide decomposition.

Several such measurements were carried out with growing cultures containing ethanol as the oxidizable component. The results in all instances showed that formic acid is decomposed somewhat more slowly than carbon dioxide. The molecular ratio of formic acid to carbon dioxide decomposed varied from 0.23 to 0.81. Data from a typical experiment are given in Fig. 1. Medium B containing 0.18 per cent sodium formate was used.
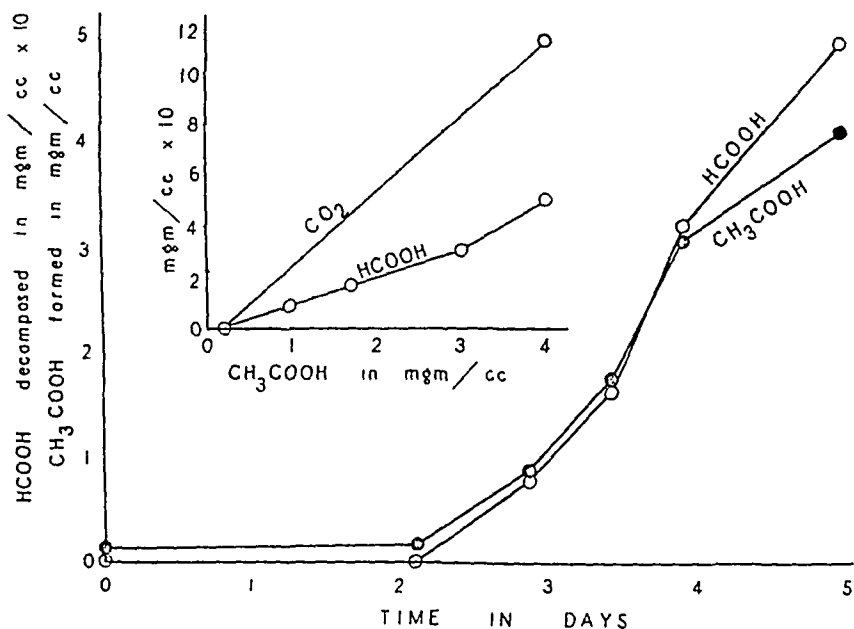
Fig. 1. Relations among carbon dioxide decomposition, formic acid decomposition, and acetic acid formation in a growing culture of *Methanobacterium omelianskii*.

It can be seen in Fig. 1 that formic acid decomposition follows much the same time curve as does acetic acid formation; the two chemical changes are in fact almost proportional to one another. This is emphasized in the inset in Fig. 1, where the amount of formic acid decomposed is plotted as a function of the acetic acid formed. An approximate curve for carbon dioxide uptake is included for comparison. In this experiment the molecular ratio of formic acid to carbon dioxide decomposed was 0.42.

In some experiments the proportionality between formic acid decomposition and acetic acid formation was not quite so close,

the formate tending to decompose relatively more rapidly in the later stages of the fermentation.

It should be emphasized that although the above experiments show that formic acid can be converted into methane, they do not provide any evidence as to the nature of the primary reaction which formate undergoes. This could be either an oxidation or a reduction. The question of the mechanism of formate decomposition will be considered in a later communication.

Toxicity of Nitrate—It has already been mentioned that the development of *Methanobacterium omelianskii* is prevented by 0.3 per cent potassium nitrate. In order to confirm this result and to find the lowest concentration of nitrate that will just inhibit growth, two further experiments were carried out.

In the first experiment, the following KNO_3 concentrations were added to Medium B, 0, 0.05, 0.10, 0.20, and 0.4 per cent. Rapid and abundant growth occurred without nitrate. With nitrate, inhibition was very marked at 0.05 per cent and was almost complete at 0.10 per cent. At higher concentrations there was no development after 7 days.

A second experiment showed that the limiting KNO_3 concentration for growth is about 0.08 to 0.10 per cent. Below 0.02 per cent nitrate no inhibition was observed.

Assimilation of Carbon

It has been shown that the oxidation of ethanol by *Methanobacterium omelianskii* may be represented approximately by Equation 2. As has previously been mentioned, this equation is not an exact description of the over-all reaction carried out by this organism because the synthesis of cell material is disregarded. From the point of view of the vital activities of the organism, the synthesis of cell material is naturally of the first importance.

Because of the physiological peculiarities of the methane bacteria, their synthetic or assimilatory activities have a special interest. Microorganisms are commonly divided into two large physiological groups on the basis of the types of carbon compound from which they build their cell material. One group, including the chemoautotrophic and photoautotrophic bacteria, synthesizes all cell materials from carbon dioxide. The other group, compris-

ing all chemoheterotrophic organisms, appears[1] to assimilate mainly the carbon of organic compounds.   There is also a third group of organisms, the photoheterotrophic bacteria (Athiorhodaceae and Thiorhodaceae), that probably assimilates directly[2] large amounts of both organic carbon and carbon dioxide carbon. Now the methane-producing bacteria, like the photoheterotrophic bacteria, combine certain characteristics of both heterotrophic and autotrophic metabolism in that they are able both to oxidize organic compounds and to reduce carbon dioxide.   It must be emphasized at once, however, that the ability to utilize both organic compounds and carbon dioxide is not in itself any proof that these bacteria assimilate carbon from both sources.   It is quite possible that only one of the two substances serves as a source of cell material, the other fulfilling some other essential function, such as acting as an oxidizing or reducing agent.

In the special case of *Methanobacterium omelianskii* there are at least three possibilities for the synthesis of cell material.   The carbon for cell synthesis might come from ethanol alone, from carbon dioxide alone, or from both compounds.

Before the evidence in favor of one or the other of these sources of carbon is considered, it will be desirable to present data showing the magnitude of cell synthesis, and the relation between synthesis and the dissimilation reaction expressed by Equation 2.

To determine the quantity of cell synthesis, Medium B, containing ammonium sulfate as the sole nitrogen source, was used. As growth occurs in this medium, ammonia nitrogen is converted into organic nitrogen in the cells.   This cell nitrogen can be measured as the difference between total (Kjeldahl) nitrogen and ammonia nitrogen.   The nitrogen determinations were carried out directly on 10 cc. aliquots of the fermented culture medium by micromethods.   Suitable blank corrections were made on the reagents and the culture medium.

Several series of cell nitrogen determinations were carried out. In each series the amount of bacterial growth in individual cultures

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[1] Recent work by Wood and Werkman (6) and others indicates that carbon dioxide may be utilized by various heterotrophic bacteria.   Ruben *et al.* (7, 8) have proved with the aid of radioactive carbon that small amounts of CO_2 are assimilated by many heterotrophic organisms.

[2] Private communication from Professor C. B. van Niel.

was varied over a considerable range by adding different initial quantities of sodium carbonate, the carbon dioxide supply being limiting. Acetic acid as well as cell nitrogen was determined on each culture after growth and fermentation had ceased. The combined results of several series of determinations are shown in Fig. 2, where cell nitrogen is plotted as a function of the quantity of acetic acid formed. The latter, as has been previously shown
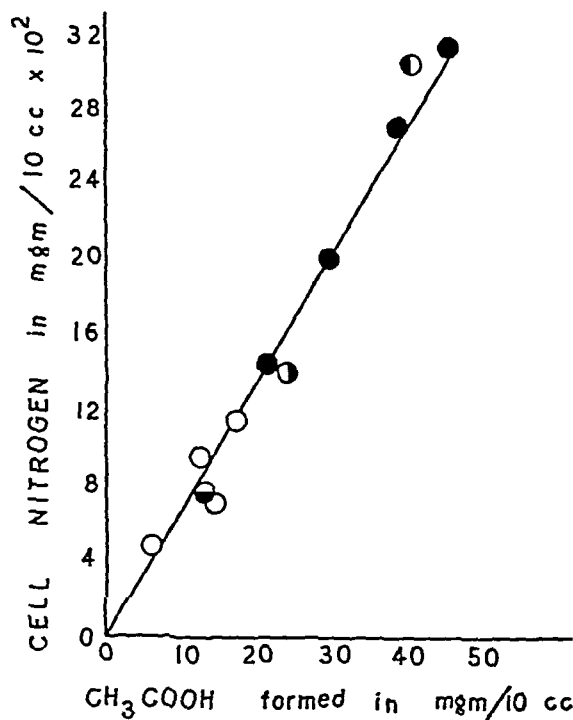
FIG. 2. Relation between cell nitrogen and acetic acid formation

(3), is a direct measure of the amount of ethanol and carbon dioxide converted.

Fig. 2 shows that under the conditions chosen the quantity of cell nitrogen is a linear function of the acetic acid formed. This linear function is of considerable interest in itself, for it shows that in anaerobic as well as in aerobic organisms catabolism and anabolism are closely connected if not completely interdependent

processes. From the slope of the line it can be calculated that 6.75 mg. of cell nitrogen are formed per gm. of acetic acid.   Since the acetic acid in such a fermentation accounts for only about 94 per cent of the alcohol oxidized (Table I), 8.2 mg. of cell nitrogen are formed per gm. of ethanol decomposed.

From the above data on nitrogen assimilation, the quantities of carbon assimilated can be calculated provided the ratio of carbon to nitrogen in the bacterial cells is known.   This ratio has been estimated approximately by determining the nitrogen content of cells grown under conditions comparable to those existing in the above experiments.   The nitrogen content was found to be 10.6 per cent on a dry weight, ash-free basis.   Assuming a carbon content of 50 per cent (which can hardly differ by more than 2 to 3 per cent from the true value), the C:N ratio would be 4.7. The carbon assimilated is therefore 38.6 mg. per gm. of ethanol decomposed, 74 mg. per gm. of ethanol carbon, or 59 mg. per gm. of substrate (ethanol plus carbon dioxide) carbon.

Because of the rather large quantity of cell material formed, it appeared possible to decide by analytical methods which of the three sources of cell carbon mentioned above is actually utilized. For if the cell material originates exclusively from ethanol, the carbon of the recovered acetic acid should be approximately 7 per cent less than the carbon of the alcohol decomposed, while the carbon dioxide and methane carbons should balance.   If, on the contrary, the cell material is derived only from carbon dioxide, the methane carbon should be about 28 per cent less than the carbon dioxide carbon, while the ethanol and acetic acid carbons should balance.   Or, finally, if both ethanol and carbon dioxide contributed to the cell material, there should be a carbon discrepancy in both the oxidation and the reduction reactions.

In order to obtain significant evidence on the source of cell carbon by the method indicated, it was necessary to carry out analyses of a rather high order of accuracy.   The over-all analytical error should, if possible, not exceed 2 to 4 per cent, whereas in most analyses of the type required the over-all error is considerably greater, usually 4 to 8 per cent.   Fortunately in the present case, the medium to be analyzed was sufficiently simple so that nearly the desired accuracy could be attained by giving careful attention to details of procedure.

The results of three carbon balance experiments will be presented. Analytical data on the transformations of ethanol, acetic acid, carbon dioxide, and methane in these experiments have already been given in Table I (Experiments 1, 2, and 3). The data show that the yield of acetic acid is always too low to account for the ethanol oxidized, whereas in two of the three experiments (Nos. 1 and 2) the quantity of methane corresponds very closely to the carbon dioxide reduced. In Table III the quantities of cell carbon formed are given and are compared with the differences between ethanol carbon and acetic acid carbon and between carbon dioxide carbon and methane carbon.

When the data given in Table III are evaluated, it must be remembered that the figures, except those for cell carbon, represent

TABLE III

Carbon Assimilation by Methanobacterium omelianskii

Carbon fraction	Experiment 1		Experiment 2		Experiment 3	
	mg	per cent	mg.	per cent	mg.	per cent
Cell C..	113	100	70	100	115	100
C_2H_5OH-C less HOAc-C	110	97	41	59	95	82
CO_2-C less CH_4-C	2	2	4	6	33	29
Total cell C accounted for	112	99	45	65	128	111

small differences between values of a higher order of magnitude and are therefore subject to considerable errors. The data nevertheless strongly indicate that the major portion of the cell carbon is derived from ethanol. In Experiments 1, 2, and 3, 97, 59, and 82 per cent of the cell carbon, respectively, is accounted for as unrecovered ethanol carbon. The percentage of cell carbon apparently derived from carbon dioxide in Experiments 1 and 2 is very small, 2 and 6 per cent, while in Experiment 3 it is 29 per cent.

It seems reasonably safe to conclude from the results of these experiments that approximately 60 per cent or more of the cell carbon is derived from ethanol, while only 30 per cent or less is derived from carbon dioxide. In other words, cell carbon definitely is not derived from carbon dioxide alone. The experiments are not sufficiently accurate to determine whether cell carbon

originates exclusively from ethanol or mainly from ethanol and to a lesser extent from carbon dioxide.

Fortunately, a more sensitive method was available for determining whether or not carbon dioxide is assimilated by *Methano-bacterium omelianskii;* namely, a method involving the use of radioactive carbon. If suspensions of cells are provided with ethanol and radioactive carbon dioxide, it should be possible to find out whether and to what extent carbon dioxide is assimilated by measuring the radioactivities of the methane evolved and of the residual cell material. This has been done and it has been found (2) that a small part (about 1.5 per cent) of the carbon dioxide reduced is converted into a non-volatile substance associated with the cells. The quantity of this non-volatile material is too small to have been detected with certainty in the analytical experiments described above. Though the percentage of carbon dioxide carbon converted into cells appears to be definitely lower than the percentage of ethanol carbon so converted, still the two are of approximately the same order of magnitude. The organism appears therefore to have no very decided preference as to which form of carbon it uses.

SUMMARY

1. The ability of a pure culture of *Methanobacterium omelianskii* to oxidize a number of organic compounds (with carbon dioxide as the ultimate oxidizing agent) has been tested. Of the compounds studied only simple primary and secondary alcohols are attacked, these being oxidized to the corresponding fatty acids and ketones, respectively. Carbon dioxide is not produced in detectable amounts in these oxidations but is, on the contrary, consumed in an amount required by Equation 1, $4H_2A + CO_2 \rightarrow 4A + CH_4 + 2H_2O$.

2. When the behavior of the organism toward various possible oxidizing agents was examined, it was found that oxygen and nitrate are toxic, sulfate and methanol are inert, and only formate is decomposed.

In growth experiments formate is only utilized when both ethanol and carbon dioxide are simultaneously present; *i.e.,* formate cannot replace either ethanol as a hydrogen donor or carbon dioxide as a hydrogen acceptor. It is concluded that

formate is not a normal intermediate in the reduction of carbon dioxide to methane in spite of the fact that formate is converted into methane. The primary reaction of formate is not considered here.

3. A study of the assimilation of carbon by *Methanobacterium omelianskii* shows that there is a linear relation between the amount of ethanol oxidized to acetic acid and the amount of cell material formed. For each gm. of substrate (ethanol plus carbon dioxide) carbon converted about 60 mg. of carbon are assimilated. By means of carbon balance experiments and by the use of radioactive carbon dioxide it is shown that both ethanol carbon and carbon dioxide carbon are converted into cell material. Most of the cell carbon is derived from ethanol. But the percentage of carbon dioxide carbon converted into cells appears to be of the same order of magnitude as the percentage of ethanol carbon so converted.

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POLYHYDROXYANTHRAQUINONES AFFECTING COAGULATION TIME IN VITAMIN K DEFICIENCY

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Following the report of Almquist and Klose (1) on the anti-hemorrhagic activity of phthiocol, 2-methyl-3-hydroxy-1,4-naphthoquinone, numerous reports have appeared on the activity of various other naphthoquinones (2–6). Vitamin K activity in the benzoquinone series was reported by Ansbacher and Fernholz (7). Of the anthraquinone series, few members have been tested; anthraquinone and 1,2-dihydroxyanthraquinone were tested by Almquist and Klose (8) and phenanthraquinone, anthraquinone-sulfonic acid, and dihydroanthraquinone diacetate by Thayer et al. (2), and all reported negative findings.

In the course of studies of vitamin K, we have tested certain new quinones and retested some of those already reported in the literature. The method of testing was that suggested by Ansbacher (9). Approximately 1000 chicks were used in these tests, usually forty chicks to a compound. Clotting times in these vitamin K-deficient chicks are frequently in excess of 24 hours. The chicks were eliminated from use in testing compounds unless the clotting time exceeded 6 hours. Normal clotting times were found to be universally less than 10 minutes, averaging 4 to 5 minutes. Where possible, each compound was tested at several different levels up to 100 mg. Table I summarizes the results obtained.

We rechecked the activity of purpurin, using prothrombin times according to the technique of Quick (10) as modified by Almquist and Klose (11). On a series of thirty-five vitamin K-deficient chicks we found the average prothrombin times to be 30 minutes. 6 hours after the administration of 0.1 mg. of purpurin, prothrombin time was lowered to an average of 3 minutes in a series

of forty chicks given vitamin K. Normal chicks and chicks on a vitamin K-deficient diet given an adequate dosage of 2-methyl-1,4-naphthoquinone showed prothrombin times of 20 to 30 seconds. The activity of vitamin K_1 is between 100 and 1000 times that of purpurin, the most active of the polyhydroxyanthraquinones. With 2-methyl-1,4-naphthoquinone as a standard, purpurin has 10,000 units per gm. as compared to 1,000,000 for the standard. Rufigallol, anthragallol, and duroquinone show only 100 units per gm.

TABLE I

Vitamin K Activity of Quinones

2-Methoxy-3-methyl-1,4-benzoquinone Pseudocumoquinone 2-Methyl-1,4-benzoquinone	Toxic at 10 mg. doses; inactive at 1 mg.
2,7-Dinitrophenanthraquinone Anthraquinone 2-Hydroxy-3-methylanthraquinone Phenanthraquinone	No action at 100 mg.
2,3-Dimethyl-1,4-benzoquinone p-Xyloquinone Quinalizarin	Slight or border line effect at 10 mg.
Rufigallol in 10 mg. Anthragallol in 10 mg. Duroquinone " 10 " Purpurin in 0.1 mg. 2-Methyl-1,4-naphthoquinone in 1 γ	1 unit (Ansbacher (9)) activity

It is of interest to note that in alizarin (1,2-dihydroxyanthraquinone), reported negative by Almquist and Klose (8), and quinalizarin (1,2,5,8-tetrahydroxyanthraquinone), found negative or border line by us, there are two hydroxyl groups on one ring; while all the compounds giving positive reactions have three hydroxyls on one ring, which may confer a lability on the substituted ring permitting the rupture and subsequent formation of 2,3-disubstituted 1,4-naphthoquinones similar to vitamin K.

The water-soluble sodium salt of purpurin can be administered intravenously in doses far in excess of the therapeutic level, with

no untoward manifestations.   Its water solubility should confer on it the power of being absorbed from the intestinal tract in the absence of bile salts.

SUMMARY

Purpurin (1,2,4-trihydroxyanthraquinone) and certain other polyhydroxyanthraquinones have vitamin K activity when tested upon vitamin K-deficient chicks.

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THE PREPARATION OF A CASEIN HYDROLYSATE FOR THE STUDY OF THE RELATIONSHIP BETWEEN CHOLINE AND HOMOCYSTINE*

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Using a diet containing purified amino acids in place of protein, du Vigneaud *et al.* (1, 2) found that homocystine or homocysteine, when substituted for methionine, promotes the growth of rats only when choline is also present in the diet. This finding was interpreted as indicating that choline serves as the donor of a methyl group to homocysteine, converting it into methionine.

Owing to the limitations imposed by the great expense of such purified amino acids, a study has been made of the preparation and use of a casein hydrolysate in which methionine was destroyed. The decomposition of this amino acid was accomplished by a modification of the procedure of Baernstein (3) for the determination of methionine. In this method concentrated hydriodic acid is utilized for the hydrolysis of protein with the cleavage of methionine into methyl iodide and the thiolactone of homocysteine. Since the latter compound was shown by Rose and Rice (4) to support growth in the absence of methionine, when the vitamin B complex was fed in a not too highly purified form, it was expected that the hydrolysate would promote growth upon the addition of choline. However, the technique to be described resulted in a mixture of amino acids which was grossly deficient not only in methionine but also in the products of its demethylation. This is not undesirable, since it permits the preparation of diets free from both methionine and its precursors. Although

tryptophane was the only other indispensable amino acid absent from the hydrolysate, growth was improved by the addition to the diet of small amounts of *dl*-threonine (0.4 per cent) and *l*-histidine (0.2 per cent). However, in the growth experiments described threonine and histidine were not added.

<div align="center">EXPERIMENTAL</div>

Casein (100 gm.) was dissolved in hydriodic acid of specific gravity 1.7 (400 cc.) to which hypophosphorous acid (0.2 to 0.5 per cent) was added. The mixture was refluxed for 6 hours in a bath of Wood's metal, while a slow stream of nitrogen was passed through the reaction vessel and reflux condenser. Water at 50–60° was conducted through the condenser, although this may not be necessary (5). At the end of the period of hydrolysis the flask was connected with a condenser for distillation and the unbound hydriodic acid and water were removed as completely as possible *in vacuo* at a bath temperature not exceeding 120°. The residue was taken up in water and the iodide precipitated completely by agitation with silver carbonate in a darkened room; excess silver ions were removed exactly with dilute hydriodic acid. The precipitate of silver iodide was removed and washed repeatedly with hot dilute hydriodic acid and finally with hot water to remove amino acids. After the removal of iodide from the combined washings in the same manner as described above, the second precipitate of silver iodide was washed once with hot dilute hydriodic acid and several times with hot water. The small amount of iodide in these washings was precipitated as silver iodide by the addition of a dilute solution of silver acetate and discarded without washing. The combined solutions of amino acids were concentrated to a small volume, dried to a paste in an oven at 100–110°, and subjected to vacuum desiccation over phosphorus pentoxide for 18 to 24 hours at 100–110°. The material was then powdered and a small amount of sodium bicarbonate was added to neutralize the calculated amount of residual hypophosphorous acid or its products.

The complete diet, which had the composition indicated in Table I, was fed *ad libitum*. The average daily food consumption and weight change during each experimental period are shown in Table II.

The growth curve of Rat 1 (Fig. 1) shows that the hydrolysate was markedly deficient in tryptophane as well as methionine, since both amino acids were necessary for growth. The gross deficiency of the hydrolysate in methionine or derivatives of homocysteine is shown by the marked weight loss of Rat 2a when its diet was supplemented only with tryptophane and choline chloride; however, addition of homocystine resulted in a prompt

TABLE I
Composition of Basal Diet

Constituents	Per cent
Casein hydrolysate	21
Powdered sucrose	16
Dextrin (thrice extracted with alcohol) .	25.9
Salt mixture*..........	4
Agar	2
B complex concentrate† .	1
Percomorph liver oil‡. . .	0.1
Corn oil (Mazola) .	15
Beef fat	15

* Salt Mixture 1, u.s.p. XI.

† The vitamin B complex concentrate was prepared in a manner similar to that described by du Vigneaud *et al.* (2); ryzamin-B was treated with ammonium reineckate solution to remove choline, and then 10 mg. each of thiamine chloride, riboflavin, and nicotinic acid (donated by Merck and Company, Inc.) were added to each 10 gm. of the modified ryzamin-B. The ryzamin-B (a rice polishings concentrate) was generously supplied by Burroughs Wellcome and Company, through the courtesy of Dr. Robert C. Page.

‡ The percomorph liver oil (Mead Johnson and Company) contained 50 per cent cod liver oil. Each gm. of oil contains not less than 60,000 units (u.s.p. XI) of vitamin A and 8500 units (u.s.p. XI) of vitamin D.

gain in weight. The striking effect of choline on the utilization of homocystine for growth is demonstrated by the marked weight loss following the omission of this compound from the diet and the prompt growth following its replacement (Rat 2a). It is clear that in these experiments with hydriodic acid hydrolysates of casein, as with mixtures of purified amino acids, choline renders homocystine utilizable for growth purposes.

The growth curve of Rat 2b shows that the lower homologue of

<div align="center">

TABLE II

Food Consumption and Weight Change of Experimental Animals

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Rat No. and sex	Days	Supplements* added to basal diet	Average daily food consumption	Average daily change in weight
			gm.	*gm.*
1 ♀	0– 6	Methionine, 0.8%, + choline chloride, 0.5% (without tryptophane)	3.5	−1.6
	6– 7	As above + tryptophane, 0.25%	2.3	−3.1
	7–27	" " + " 0.25%	3.9	+0.4
2a ♂	0– 9	Choline chloride, 0.5%	2.5	−1.3
	9–16	Homocystine, 1%, + choline chloride, 0.5%	2.9	+0.8
	16–21	" 1%	3.1	−1.0
	21–27	" 1%, + choline chloride, 0.5%	3.9	+1.4
2b ♂	0– 5	" 1%, + S-methylcysteine, 1.5%	2.3	−0.8
	5–12	" 1%, + " 1.5%, + choline chloride, 0.5%	2.9	+0.9
	12–15	Homocystine, 1%, + β-methylcholine chloride, 0.55%	3.0	−2.0
	15–19	Homocystine, 1%, + arsenocholine chloride, 0.7%	3.7	+2.3
	19–25	Homocystine, 1%, + arsenocholine chloride, 0.7%	4.2	−0.5
	25–33	Homocystine, 1%, + arsenocholine chloride, 0.7%, + betaine hydrochloride, 1.0%	4.3	+1.2
3 ♂	0– 5	Basal diet alone	2.5	−0.3
	5–11	Homocystine, 1%, + arsenocholine chloride, 0.7%	2.4	−0.4
	11–21	Homocystine, 1%, + arsenocholine chloride, 0.7%, + choline chloride, 0.5%	3.3	+0.6
	21–26	Homocystine, 1%, + methyldiethyl-β-hydroxyethyl ammonium chloride, 0.6%	2.4	−0.4
4 ♂	0– 5	Choline chloride, 0.5%	2.6	−0.3
	5–11	Homocystine, 1%, + arsenocholine chloride, 0.7%	2.5	−0.4
	11–16	Homocystine, 1%, + arsenocholine chloride, 0.7%, + choline chloride, 0.5%	3.8	+0.5
	16–21	Homocystine, 1%, + methyldiethyl-β-hydroxyethyl ammonium chloride, 0.6%	2.7	−0.5

* Except in the diet of Rat 1, tryptophane, 0.25 per cent, was added to the diet in all cases.
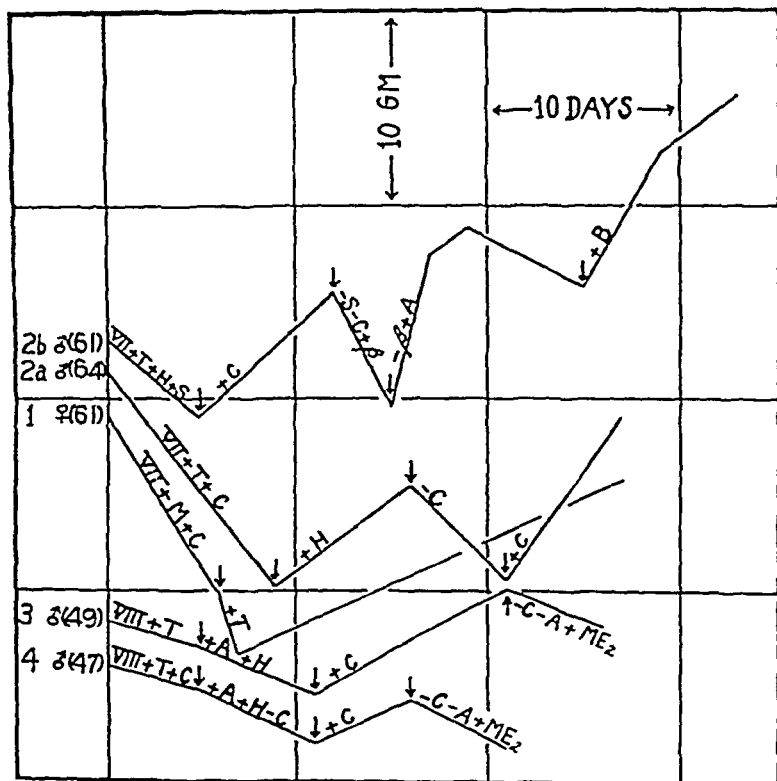
FIG. 1. The number and sex of the rat are shown on the left. The initial weight of the rat is given in parentheses. The hydrolysate fed throughout each experiment is indicated by a roman numeral at the beginning of each growth curve. Supplements to the hydrolysates are indicated by the following code: T = tryptophane, 0.25 per cent; M = methionine, 0.8; H = homocystine, 1.0; S = S-methylcysteine, 1.5; C = choline chloride, 0.5; B = betaine hydrochloride, 1.0; A = arsenocholine chloride, 0.7; ME_2 = methyldiethyl-β-hydroxyethyl ammonium chloride, 0.6; β = β-methylcholine chloride, 0.55 per cent; $+$ = addition to the diet; the minus sign = removal from the diet. The arrows indicate the points of change.

methionine, S-methylcysteine,[1] does not transfer its methyl group to homocysteine to form methionine, although in its

[1] S-Methylcysteine was prepared by the writer according to the dimethyl sulfate procedure of du Vigneaud, Loring, and Craft (6); the nitroprusside and cyanide-nitroprusside reactions were negative.

presence growth promptly follows the addition of choline to the diet. The β-methyl derivative of choline chloride[2] also failed to support growth on a homocystine diet when it was substituted for choline in the diet (Rat 2b). Although replacement of the β-methyl compound (Rat 2b) by arsenocholine chloride[3] resulted in a period of temporary growth, this was followed by a loss of weight, until betaine hydrochloride was also added to the diet, when growth was resumed. Addition of the arsenic analogue of choline chloride and of homocystine to the diet of litter mates (Rats 3 and 4) (during the previous 5 day period one had received no choline, the other 0.5 per cent choline) resulted in continued weight loss in both, until choline was also added to the diets. The ineffectiveness of the methyldiethyl homologue of choline chloride[4] as a substitute for choline is also shown (Rats 3 and 4).

DISCUSSION

The usefulness of the method described for the preparation of diets free from methionine and growth-promoting products of its demethylation is obvious, while the disadvantages are slight. The hydriodic acid employed in the hydrolysis of the casein is expensive, but much of it is recovered by vacuum distillation from the hydrolysate. Recovery of silver from silver iodide permits a further reduction in the cost of the procedure. The expense of the final diet is very much less than that of a diet containing purified amino acids purchased on the open market. The technical manipulations involved in the preparation of the hydrolysate are insignificant in comparison with those requisite to the preparation of the many amino acids used in dietary mixtures.

The finding that the casein hydrolysate contained no derivative of homocysteine which permits growth in the presence of choline was unexpected. However, the manner in which the thiolactone of homocysteine was lost has not been determined. Extraction of the silver iodide precipitated during the removal of hydriodic

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[2] Thiamine chloride, riboflavin, nicotinic acid, and β-methylcholine chloride were freely donated by Merck and Company, Inc.

[3] The samples used were supplied by Hoffmann-La Roche, Inc., to whom the writer is indebted.

[4] Prepared by treatment of diethylaminoethanol with methyl iodide and conversion of the product to the chloride with silver chloride.

acid was sufficiently thorough to prevent the loss of more than traces of homocysteine derivatives, and the degradation of the thiolactone is not likely to have followed the use of a much smaller amount of hydriodic acid per gm. of casein than is used in the determination of methionine. It is conceivable that the thiolactone may undergo decomposition as the result of a catalytic effect of the silver ions used in the removal of hydriodic acid.

The results of the feeding experiments confirm the observations of du Vigneaud and his group with regard to the dietary significance of choline (or betaine) for the growth of rats on diets containing homocystine in place of methionine. The finding that the lower homologue of methionine, S-methylcysteine, is incapable of supplying a methyl group to homocysteine was not unexpected in view of the fact that it does not support growth in rats on a cystine-deficient diet (6, 7), although the sulfur is readily oxidized by the rabbit (6) and by man (8). Further evidence that the compound is not readily demethylated has been presented by Brand and coworkers (8), who were unable to obtain a positive nitroprusside test when S-methylcysteine was shaken with kidney slices.

The temporary growth effect produced by arsenocholine in one experiment is believed not to be indicative of a methylation of homocystine, since other experiments indicate that the analogue is ineffective, a finding agreeing with that recently reported by du Vigneaud (9). It is possible that the presence of the arsenic nucleus imposes an insuperable impediment to the enzymatic process involved in the removal of methyl groups, although the structure and spatial configuration of the arsenocholine cation are such as to permit it to replace choline as a lipotropic factor (10–12), as a preventive[5] of the hemorrhagic kidney condition described in young rats by Griffith and Wade (13–15), and as a preventive[6] of perosis in young turkeys (Jukes (16)).

The triethyl homologue of choline chloride is unable to support growth on a homocystine diet (2), although it is a potent lipotropic agent (17, 18) and prophylactic against hemorrhagic kidneys.[5] This finding is particularly significant to the theory that choline serves as a source of methyl groups. The methyl-

[5] Welch, A. D., unpublished research.
[6] Jukes, T. H., personal communication.

diethyl homologue of choline, however, might be expected to function as the donor of a methyl group. That this is not the case is shown in the experimental portion, a finding recently reported by du Vigneaud also (9). This worker[7] has found the other intermediate between choline and its triethyl homologue, the ethyldimethyl homologue, to be capable of supporting growth on a homocystine diet.

A possible explanation for the inactivity of β-methylcholine as a lipotropic agent (10, 11) and as a donor of methyl groups (Fig. 1) is that the *secondary* alcoholic hydroxyl group of this substance, unlike the *primary* alcoholic hydroxyl group of choline, may not be phosphorylated efficiently by the organism. Among the numerous compounds related to choline which possess lipotropic activity only betaine and betaine aldehyde lack a primary alcoholic hydroxyl group and evidence that these compounds may serve as choline precursors has been presented by Welch and Welch (10) and by Jukes.[6] In order to promote growth on a homocystine diet choline derivatives must satisfy even more rigid requirements than for action as lipotropic agents, since in addition to the presence of a primary alcoholic hydroxyl group, at least two methyl groups attached to a nitrogen nucleus must be present.

SUMMARY

A method is described for the preparation of a hydriodic acid hydrolysate of casein which is grossly deficient in tryptophane, methionine, and growth-promoting derivatives of homocysteine. The hydrolysate serves as a relatively inexpensive substitute for mixtures of purified amino acids for the study of the relationship between choline and homocystine.

The discovery of du Vigneaud and his group that choline or betaine must be supplied to enable the rat to utilize homocystine

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[7] du Vigneaud, V., personal communication.

for growth is confirmed, as is the report that the arsenic analogue of choline chloride is ineffective in this respect. The lower homologue of methionine, S-methylcysteine, the β-methyl derivative, and the methyldiethyl homologue of choline chloride are also ineffective in inducing growth on a diet containing homocystine in place of methionine.

The significance of these results is discussed.

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HEPATIC GLYCOGEN FORMATION BY THE ISOMERS OF ALANINE

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Alanine is well recognized as a good glycogen former. The relative ability of its isomers to form glycogen is of interest because of the belief held by many that only the natural isomer of many compounds may be metabolized. Abderhalden and Tetzner (1) have reported that when *dl*-alanine is administered only the natural or *l*(+) form is utilized, the *d*(−) form failing to undergo metabolism. Butts *et al.* (2) have found that *l*(+)-alanine is approximately twice as effective a glycogen former as *dl*-alanine. This failed to coincide with our conclusion (3) that these compounds were equally good antiketogenic agents. A possible explanation was suggested by the observation (4) that the rate of glycogen formation from glycine and *dl*-alanine is quite different, although they are equally good glycogen formers. There might simply be a difference in the rate of glycogen formation by the two isomers of alanine. The observations of Wilson and Lewis (5) pointed to this possibility. Slower utilization of *d*(−)-alanine might be due to an added step in its utilization, even conversion to the natural isomer (6), before further use by the body.

Methods

Albino mice weighing 18 to 22 gm. were used. Each mouse received 0.8 cc. of a solution containing 2 moles of alanine[1] per liter. This was given through a blunt 20 gage hypodermic needle

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[1] The *dl*- and *l*(+)-alanines were the same preparations we have used before (3). The *d*(−)-alanine was obtained from F. Hoffmann-La Roche and Company, A. G., Basle, Switzerland. $[\alpha]_D^{25} = -9.6°$ for the hydrochloride in aqueous solution. The preparation contained less than 0.1 per cent of moisture and negligible ash.

which was used as a stomach tube. 99 female mice were used in the first experiment, three of them being sacrificed every 2 hours after administration of the racemic compound and each isomer and the amount of glycogen in the livers determined. In Experiment 2 a total of 240 male mice was used, groups of five mice being sacrificed every 2 hours after the administration of each compound. The last group was given the same amount of alanine as the rest,
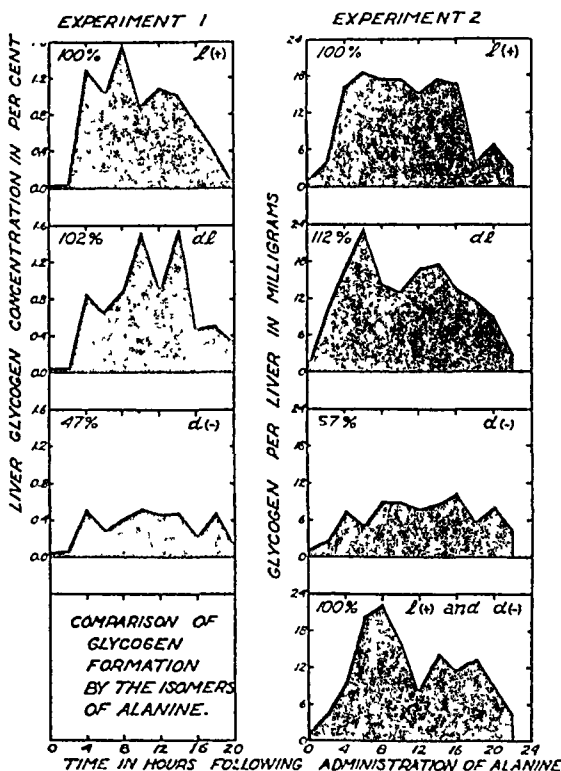
FIG. 1

equal quantities of d- and l-alanine solutions being mixed immediately before administration. Liver glycogen determinations were carried out according to the method of Good, Kramer, and Somogyi (7).

Results

Data based upon the group averages are presented in Fig. 1. In Experiment 1 the amount of glycogen is given as the concentra-

tion in the livers and in Experiment 2 as the amount per liver. Similar conclusions may be reached from either manner of presenting the results. The percentage figures given with each curve represent the relation of the curve areas in a given experiment to the area of the $l(+)$-alanine curve taken as 100 per cent.

The data show very clearly that dl-alanine is just as good a hepatic glycogen former as the natural isomer, $l(+)$-alanine. A freshly made mixture of $l(+)$- and $d(-)$-alanine solutions gives approximately the same amount of glycogen as the racemic and natural compounds. The unnatural isomer $d(-)$-alanine, on the other hand, is a much inferior glycogen former.

DISCUSSION

These results coincide with the equal antiketogenic activity of $l(+)$- and dl-alanine (3). They are at variance with other observations on glycogen formation by $l(+)$- and dl-alanine (2) but this discrepancy is undoubtedly due to the time in the absorption period at which these observations were made. Our conclusions are not in harmony with the complete conversion of $d(-)$-alanine (8) as well as dl-alanine to glucose (9) in the phlorhizinized organism after small doses by mouth. In the light of these observations as well as our finding that dl-alanine is as good a hepatic glycogen former as $l(+)$-alanine it is surprising that $d(-)$-alanine when fed alone forms glycogen so poorly. Our data show that the liver glycogen tends to be sustained at a higher level for a longer period after administration of dl-alanine than $l(+)$-alanine. This would indicate a slower rate of glycogen synthesis by the $d(-)$-alanine, which was suggested as a possibility in the introduction to this note. But with other compounds forming glycogen at different rates the amount of glycogen ultimately formed is the same in every case (4). The reason for the better glycogen-forming ability of $d(-)$-alanine when fed as a constituent of the racemic form than when fed alone is not entirely clear.

The most likely explanation of our results would appear to us to be as follows: When fed alone, $l(+)$-alanine, the natural form, is absorbed, deaminized, and rapidly converted in whole or in large part to glycogen by the liver. The $d(-)$-alanine on the other hand is probably absorbed at a not very different rate than

is the $l(+)$ form (10) but is[2] utilized much more slowly by the organism (1).   As a result a large proportion of the $d(-)$-alanine is lost in the urine when a large dose of this isomer is fed alone. When dl-alanine is fed, the $l(+)$ isomer is used rapidly and the $d(-)$ isomer is used slowly, just as when each is given alone.   But in this case the isomers are fed in only half the quantities given when the active compounds are fed.   During the entire absorption period the organism need convert to glycogen only 50 per cent of the $d(-)$-alanine offered the liver when $d(-)$-alanine is fed. This does not tax the mechanism available to the organism for using $d(-)$-alanine or converting it to the $l(+)$ form with the subsequent ready use of this isomer, and little or no $d(-)$-alanine is lost in the urine.   This reasoning would explain the results of Abderhalden and Tetzner (1), for these investigators injected the alanine subcutaneously and the organism must have been flooded with the isomers for a relatively short period.   With its slow utilization rate only a small amount of the $d(-)$ isomer could be expected to escape excretion by the kidneys and this is what they found.   It would also explain the complete conversion to glucose of $d(-)$-alanine (8) as well as dl-alanine when fed in small doses to the phlorhizinized dog.

We are well aware that there is no way in which the total amount of glycogen formed by a metabolized substance may be determined with certainty by measurements of liver glycogen.   Estimations

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[2] The most reasonable conclusion one may reach from Abderhalden and Tetzner's (1) results considered together with our data is that $d(-)$-alanine is utilized much more slowly than the $l(+)$ form.   Our results indicate that the maximum rate of conversion of $d(-)$-alanine to glycogen is definitely limited.   In earlier experiments we found that when large doses of $l(+)$-alanine were fed to the rat about 5 per cent was lost in the urine, while after $d(-)$-alanine 27 per cent was lost, if all of the extra amino nitrogen was assumed to be alanine.   In neither case was the form or optical activity of the amino nitrogen compound which was excreted determined. These results would suggest the same conclusion as has been reached above.   It has recently been found (11) that rat liver slices deaminate $d(-)$-alanine more rapidly than the natural $l(+)$ form, which is contrary to the idea we have expressed here.   The metabolism of such unnatural groups of cells in an unnatural environment may be quite different from that which exists in the complex relationships of the intact organism. Consequently we are hesitant in drawing broad conclusions from such results except when they confirm data obtained from the intact organism.

of glycogen formation from the concentration in the liver involve the assumption that the liver content is proportional to the total catabolism of the compound being studied. This method has been widely used and probably has a reasonable basis when, as in this case, the compounds being compared are very similar in nature.

SUMMARY

In equimolecular doses the racemic *dl*-alanine forms as much hepatic glycogen as does the natural isomer *l*(+)-alanine. In the same dose *d*(−)-alanine is a much inferior glycogen former. It is suggested that *d*(−)-alanine is a better glycogen former when fed as a part of the racemic mixture than when fed alone, because the amount absorbed per hour is greatly reduced and this unnatural isomer is converted slowly and at a definitely limited rate.

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SPECIFIC AND NON-SPECIFIC CELL POLYSACCHARIDES OF AN AVIAN STRAIN OF TUBERCLE BACILLUS*

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Previous communications from this laboratory have dealt with the complex mixture of polysaccharides extracted from defatted cells of a human strain, H-37 (1–3), and from the defatted cells of a bovine strain, M-1698 (4), of the tubercle bacillus. These polysaccharides were extracted by methods in which the use of heat, alkali, and mineral acid was avoided. This work has now been extended to include the cell polysaccharides of an avian strain, No. 531, of the tubercle bacillus.[1]

The methods employed were essentially those used in the previous work. Modifications are described in detail.

EXPERIMENTAL

600 gm. of dried, defatted, and ground avian tubercle bacilli, Strain 531, were shaken with chloroform and filtered by suction on a large Buchner funnel. The gummy solid was ground in a mortar with alcohol, refiltered, and washed with acetone by gravity. The acetone was removed by suction. The dry bacterial mass, freed of large amounts of wax by this procedure, was wet with 1 liter of 3.5 per cent acetic acid, ground to a thin slurry in a mortar, and placed in a percolator provided with a porcelain

filter plate covered with filter paper, asbestos, and a layer of purified sand. The mass was then percolated under slight suction with 0.35 per cent acetic acid containing 0.5 per cent phenol for 1 month, yielding 25 liters of percolate. Extraction was continued for 6 more months, during which 90 more liters of percolate were obtained. The two portions were concentrated *in vacuo* and worked up separately as Lots 554 and 555, as described in Flow Sheets I to IV. As in the previous work, all fractions were reprecipitated from the smallest possible amount of water until material of lower and higher solubility was removed. Extraction of Lot 554 with glacial acetic acid, after separation of the fraction soluble in strong alcohol, dissolved most of the colored substances with little extraction of serologically active polysaccharides and obviated the necessity for treatment with cupric acetate (4). Proteins were removed from the main fractions by shaking with chloroform (5). Inorganic phosphates were precipitated with uranyl acetate. Several additional treatments with chloroform and butyl alcohol were found to be effective in removing small amounts of colloidal uranyl phosphate. After fractionation the polysaccharides were precipitated from aqueous solution with redistilled acetone and dried to constant weight *in vacuo* over P_2O_5, NaOH pellets, and paraffin before analysis. The formation of colloidal solutions on the addition of acetone could generally be avoided by treatment of the aqueous solutions with 4 to 5 volumes of methyl alcohol before addition of acetone.

An attempt was made with Lot 555 to separate the serologically active polysaccharides by dialysis. Proteins, inorganic phosphates, and the fraction soluble in strong alcohol were removed as before. The dark aqueous solution was shaken with basic copper carbonate to remove color, after which the solution was centrifuged and dialyzed in a cellophane tube against water at slightly reduced pressure. The outside solution (No. 555 II) and the inside solution (No. 555 I) were concentrated *in vacuo* and fractionally precipitated with methyl alcohol as described in Flow Sheet IV. A total of 6.4 gm. of polysaccharide was obtained, of which 6.0 gm., or 94 per cent, were dialyzable.

Lot 554

The separation of the main fractions is indicated in Flow Sheet I, and the fractionation of Fraction 554 I into the principal

Flow Sheet I

Separation of Main Fractions of Carbohydrates from

Avian Tubercle Bacillus Cells and Fractionation of 554 A into Components

Lot 554 extract, concentrated in vacuo

Shaken with $CHCl_3$-BuOH and treated with
10 vols. of EtOH

Ppt.

Solution
554 A
(Combined with 555 A)

Dry ppt. extracted
with HOAc

Residue

Solution
554 III

Concd. in vacuo to sirup,
taken up in MeOH, then +
6 vols. EtOH

Dissolved in H_2O
and pptd. at
95% HOAc

Ppt.
A_1

Ppt.
554 I

Solution

Supernatant concd.
in vacuo to sirup,
taken up in HOAc
and pptd. with 2 vols.
acetone

Pptd. with
acetone

554 II

Ppt.
A_2

Supernatant
into 10 vols.
acetone

Ppt.
A_3

Supernatant concd.
in vacuo to sirup,
dissolved in
acetone, centrifuged
from NaOAc in the
cold, and the
acetone soln.
evapd. in vacuo

A_4

Separation of 554 I (Flow Sheet I) into Component Fractions

554 I

Dissolved in water, saliva-treated, inorganic P removed with uranyl acetate, and solution made up to 75% MeOH

Supernatant to 90% MeOH

Supernatant into acetone

Ppt. I B$_1$

to 75% MeOH

Ppt. (Combined with I B$_3$)

Supernatant to 90% MeOH

Supernatant into acetone

Ppt. I B$_4$

to 80% HOAc

Supernatant into acetone

Ppt. I B$_1$

to 80% HOAc

Ppt. MgSO$_4$

Supernatant into acetone

Ppt. I B$_1$

Super-natant into acetone

Ppt. I B$_4$

Ppt. MgSO$_4$

Ppt. I B$_2$

to 75% MeOH

Ppt. I B$_3$

Super-natant into acetone I B$_2$

to 80% HOAc

Ppt. I B$_3$C

(85% MgSO$_4$)

Super-natant into acetone

Ppt. I B$_3$

Ppt. I C

Dissolved in water, made up to 66% MeOH

Supernatant into acetone

I C$_6$

to 80% HOAc

Super-natant into acetone

I C$_6$

Ppt. MgSO$_4$

Supernatant into acetone

Ppt. I C$_4$

Ppt. I C$_1$

to 80% HOAc

Super-natant to 90% HOAc

Super-natant to 95% HOAc

Ppt. I C$_3$

to 90% HOAc

Super-natant into acetone

Ppt. I C$_1$

Ppt. I C$_2$

to 80% HOAc

Ppt. I C$_{2a}$

Super-natant into acetone

I C$_{2b}$

Ppt. I C$_{3a}$

Flow Sheet III

Separation of Fraction 554 II (Flow Sheet I) into Component Fractions

554 II

Dissolved in water, saliva-treated,
inorganic P removed with uranyl
acetate, and the solution made up
to 75% MeOH

Ppt.
II C

to 50%
MeOH

Ppt.
K₂SO₄

Supernatant to
90% MeOH

Supernatant
into
acetone

Ppt.
II C

Ppt.
II B₂

Supernatant into
acetone

to 75%
MeOH

Ppt.
II B₂C

Supernatant
into
acetone

Ppt.
II B₁

to 90%
MeOH

Ppt.
II B₂

Ppt.
II B₁C

Supernatant
into
acetone

Ppt.
II B₁
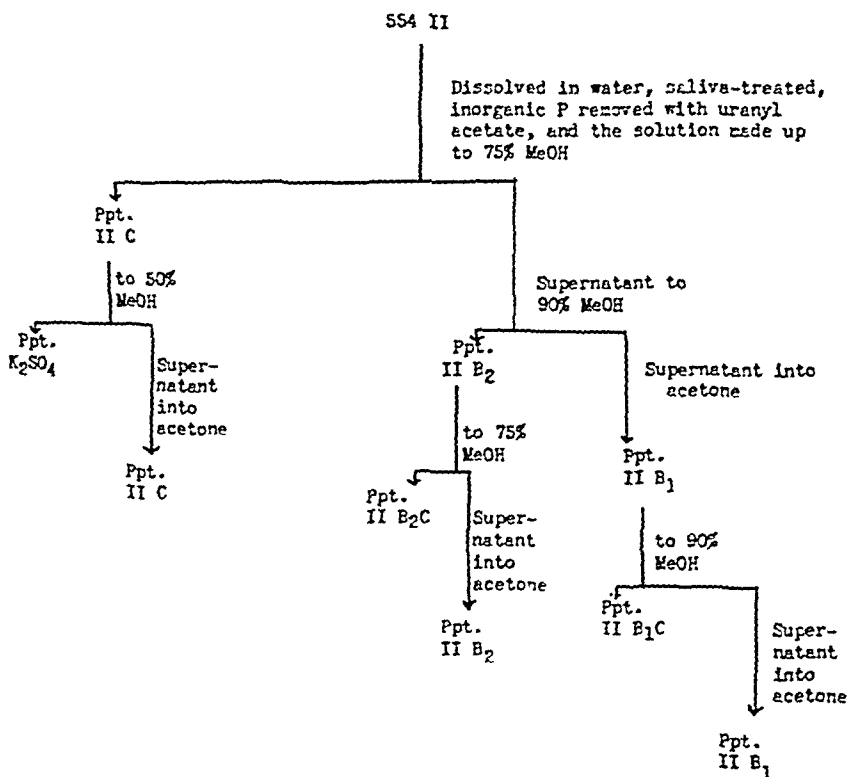
fractions, C, B_2, and B_1, corresponding to those obtained from the human strain (3), is indicated in Flow Sheet II, together with the subfractions obtained by further fractionation. The fractions soluble in 66 per cent methyl alcohol (I C_6, I B_2, and I B_1) all gave large amounts of crystalline magnesium sulfate when acetic acid was added dropwise in the cold to the aqueous solutions to 80 per cent concentration. When Fraction II C, insoluble in 75 per cent methyl alcohol (Flow Sheet III), was made up to 50 per cent by the dropwise addition of methyl alcohol in the cold, 0.7 gm. of crystalline potassium sulfate was obtained.

The main fraction, No. III (Flow Sheet I), soluble in glacial acetic acid, contained most of the color, but could be freed from this and separated into a number of fractions by methods previously described (cf. also (3, 4)). Although 3.6 gm. of these fractions were isolated, all but one contained considerable nitrogen and, as they were either inactive or only slightly active in the antisera available, further details are omitted.

12.6 gm. of fractions soluble in strong ethyl alcohol (Fractions 554 A and 555 A) were very dark in color and were relatively inactive serologically. Details are also omitted.

The yields and properties of the various fractions are included in Table I.

Lot 555

The fraction precipitable at 66 per cent methyl alcohol, Fraction I C, was found to be completely dialyzable. The II C and II B_2 fractions, which were high in ash, were refractionated several times at 80 and 96 per cent acetic acid (Flow Sheet IV). The ash was reduced approximately 50 per cent, and the serological activity increased slightly. Yields and properties of the various fractions are given in Table II.

Chemical Properties

Treatment of the main fractions with uranyl acetate removed the major portion of inorganic phosphate, and, since in no case was the total phosphorus over 1 per cent, the amount of organically bound phosphorus was small. Pentose was estimated by a modification of the Neumann test (6). Inosite was found in some of the fractions by the Scherer test, as noted in Tables I and II.

· Fraction 554 I C₁ gave clear solutions during the fractionation procedure, but highly opalescent solutions were obtained when the isolated and dried material was dissolved in water for analysis. Treatment with N sodium hydroxide in the cold for 3 hours

TABLE I

Properties of Polysaccharide Fractions of Avian Strain 531 of Tubercle Bacillus

Fraction No.	Yield	$[\alpha]_D$	$[\alpha]_{Hg}$	Neutral equivalent	N	P	Pentose	Inosito	Precipitin test*	Ash
	gm.	*degrees*	*degrees*		*per cent*	*per cent*				*per cent*
554 I C₁	2.68	+72			0.5	0.4	+	−	+ (+±)	2.1†
I C₂ₐ	0.53	+86	+98		0.7	0.3	++	±	+ (++)	1.9†
I C₂ᵦ	0.23	+88		2000	0.7	0.2	++	±	+ (++)	1.3†
I C₃ₐ	0.17	+70	+81		1.8	0.5	+	+	± (+±)	6.9
I C₃ᵦ	0.10	+53	+69	800	2.9	0.2	+	+±	+ (+±)	1.0
I C₄	0.27	+37	+49	350	6.5	0.3	+	−	− (−)	0.8
I C₆	1.01	+61	+72	1000	1.8	0.6	++	±	+ (+±)	1.2
I B₂	0.36	+67	+80	1300	1.5	0.2	+++	±	+ (++)	1.0
I B₃	0.62	+55	+64		2.1	0.9	+	+	± (+±)	3.9
I B₄	0.20	+52	+60		1.9	0.7	+	−	− (+±)	5.8
I B₁	0.51	+66	+79	1700	1.2	0.9	+++	−	+ (++)	1.4
II C	2.29	+26	+30		3.3	0.9	+	−	± (+)	12.1
II B₂	0.64	+44	+51		2.6	0.4	+	−	± (+±)	11.6
II B₁	0.31	+19	+23		4.4	0.9	±	−	− (+±)	12.1
II B₂C	1.65	+10	+14		3.2	0.9	±	±	− (±)	15.1
II B₁C	0.51	+17	+21		3.5	0.7	+	−	± (+±)	13.9
I CIₐ	0.54‡	+77	+93	4000	0.2	0.3	+		+ (+±)	0.2
I CIᵦ	0.28‡	+86	+107	6500	0.2	0.3	+		+ (++)	0.5

$[\alpha]$, N, and P are calculated to the ash-free basis.

* Precipitin tests were carried out in 1:500,000 dilution with anti-H-37 horse serum No. 5807-L. Readings after centrifugation are given in parentheses.

† Ash values marked thus were calculated as Mg, the others as Ca.

‡ Obtained from Fraction I C₁ after treatment with NaOH, I CIₐ being insoluble, and I CIᵦ soluble in 80 per cent acetic acid.

gave a small amount of white flocculent precipitate which was washed by centrifugation and treated with hydrochloric acid. The acidified solution was filtered and the precipitate was recrystallized repeatedly from methyl alcohol-water. The final

Flow Sheet IV

Separation of Lot 555 into Component Fractions

Lot 555 (aqueous solution)

Proteins removed with $CHCl_3$-BuOH, phosphate removed
with uranyl acetate, and the soln. poured into
10 vols. of EtOH

Ppt.
555

Supernatant 555 A
(Combined with 554 A)

Ppt. dissolved in H_2O, treated
with $CuCO_3 \cdot Cu(OH)_2$ and the supernatant
dialyzed

Dialysis, inside
555 I

Dialysis, outside
555 II

to 66%
MeOH

Ppt.
I C

Supernatant
to 90%
MeOH

Ppt.
I B$_2$

Supernatant
into
acetone

Ppt.
I B$_1$

to 66%
MeOH

Ppt.
II C

Supernatant to
90% MeOH

to 80%
HOAc

Ppt.
II C$_1$

Supernatant
to 96%
HOAc

Ppt.
II C$_2$

Supernatant
into
acetone

Ppt.
II B$_2$

to 96%
HOAc

Ppt.
II B$_2$

Supernatant
into
acetone

Ppt.

Supernatant
into
acetone

Ppt.

extracted
with
MeOH

Insoluble   Solubl

crystalline product melted at 55–57°. A mixture with purified palmitic acid, melting at 60.5–61.5°, also melted at 55–57°. The acid filtrate gave a strong test for magnesium. The ash content of the recovered polysaccharide fell from 2.1 per cent to 0.2 per cent, and a portion of the fraction (I CI_b) was found to be soluble at 80 per cent acetic acid (Table I).

TABLE II

Properties of Polysaccharide Fractions of Avian Strain 531 of Tubercle Bacillus

Fraction No.	Yield	$[\alpha]_D$	$[\alpha]_{H\alpha}$	Neutral equiva-lent	N	P	Pentose	Ino-site	Precipitin test*	Ash
	gm.	degrees	degrees		per cent	per cent				per cent
555 I C	0.01								± (+)	
I B₂	0.22	+81		8700	1.4	0.2	+++	+	+ (++±)	0.4
I B₁	0.13	+59			2.2	0.2	+++	+	+ (++)	3.5
II C	2.32	+44	+51		1.8	0.5	±	±	± (++)	10.7
II B₂	1.35	+57	+69		2.5	0.4	++	±	+ (++)	8.1
II B₁	0.97	+58	+66		4.0	0.3	+++±	±	+ (+±)	5.0
III	1.32	−3	−5		2.7	0.3	−	±	− (±)	15.2
II C₁†	0.79	+61	+72		0.5		++		+ (+±)	6.4
II C₂†	0.33	+28	+42		1.5		++		+± (++)	3.1
II B₂†	0.33	+45	+58		0.6		+++		+± (++)	4.1
II CB₂†	0.86	+56	+66		3.4		++		+ (+±)	5.7

$[\alpha]$, N, and P are calculated to the ash-free basis for the fractions on which ash was determined.

* Carried out at 1:500,000 dilution with anti-H-37 horse serum No. 5807-L.

† Obtained after refractionation of Fractions II C and II B₂ as described in Flow Sheet IV.

The corresponding 555 I C and II C fractions did not give opalescent solutions.

Serological Properties of Principal Fractions

All fractions were tested qualitatively with anti-H-37 horse serum, No. 5807-L, and the principal fractions were used for quantitative absorption of this serum and of pooled anti-avian rabbit sera obtained by the injection into rabbits of the dried, defatted Strain 531 of avian bacilli. The principal fractions gave positive precipitin tests at dilutions of 1:2 million in Serum

TABLE III

Quantitative Absorption of 5.0 Ml. of Anti-H-37 Horse Serum No. 5807-L with 0.10 Mg. Portions of Lot 554 Polysaccharide Fractions

Fraction No.	Nitrogen pptd.				
	1st absorption	2nd absorption	3rd absorption	4th absorption	Total
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
554 I B$_3$	0.354	0.108	0.010		0.472
I C$_{2a}$	0.362	0.086	0.006		0.454
I C$_{2b}$	0.416	0.046	0.008		0.470
I C$_{3a}$	0.112	0.058	0.028	0.030	0.228
I C$_{3b}$	0.152	0.080	0.058	0.034	0.324
I C$_4$	0.064	0.020	0.016	0.020	0.120
I B$_2$	0.506	0.012	0.002		0.520
I B$_4$	0.376	0.090	0.008		0.474
I B$_1$	0.416	0.028	0.010		0.454
I C$_1$	0.118	0.044	0.048		0.210
I C$_5$	0.356	0.096	0.010		0.462
II C	0.104	0.088	0.070	0.042	0.304
II B$_2$	0.186	0.132	0.046	0.012	0.376
II B$_1$	0.016*	0.150	0.078	0.022	0.266
II B$_2$C	0.078	0.070	0.036	0.004	0.188
II B$_1$C	0.184	0.100	0.020	0.00	0.304
III B$_{1a}$	0.158†	0.106	0.034	0.010	0.308

* Only 0.02 mg. of polysaccharide was added for the first absorption.
† One tube only.

TABLE IV

Quantitative Absorption of 3.0 Ml. of Anti-H-37 Horse Serum with 0.1 Mg. Portions of Lot 555 Polysaccharide Fractions

Fraction No.	Nitrogen pptd.			Total N pptd.	Total N pptd. per 5.0 ml. serum
	1st absorption	2nd absorption	3rd absorption		
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
555 I C	0.192	0.096	0.008*	0.296	0.493
I B$_2$	0.328	0.014		0.342	0.570
I B$_1$	0.310	0.010		0.320	0.533
II C	0.114	0.058	0.026*	0.198	0.330
II B$_2$	0.286	0.016		0.302	0.503
II B$_1$	0.244	0.010		0.254	0.423

* 0.05 mg. of polysaccharide used.

5807-L. The precipitin tests in Tables I to III are recorded only for the 1:500,000 dilution in order to emphasize differences between highly active and relatively inactive fractions. Quantitative absorptions were run on 3.0 to 5.0 ml. of Serum 5807-L and pooled anti-avian rabbit serum (Tables III to V). All analyses were run in duplicate and all manipulations were carried out in the cold, the tubes being allowed to stand for 2 days after the first addition of polysaccharide and 5 to 7 days for each succeeding absorption. Qualitative cross precipitin tests were run on the supernatants from the quantitative absorptions. For comparison

TABLE V

Quantitative Absorption of 5.0 Ml. of Pooled Anti-Avian Rabbit Sera (Rabbits 6.56 to 6.60) with 0.1 Mg. Portions of Lot 554 Polysaccharide Fractions

Fraction No.	Nitrogen pptd.					
	1st absorption	2nd absorption	3rd absorption*	4th absorption*	5th absorption*	Total
	mg.	mg.	mg.	mg.	mg.	mg.
554 I C$_1$	0.092	0.044	0.072	0.076	0.050	0.334
I C$_{2a}$	0.324	0.208	0.172	0.020		0.724
I C$_{2b}$	0.414	0.226	0.112	0.004		0.756
I C$_6$	0.308	0.192	0.088	0.026		0.614
I B$_2$	0.428	0.090	0.088	0.018		0.624
I B$_3$	0.184	0.136	0.094	0.036		0.450
I B$_4$	0.274	0.130	0.064	0.034		0.502
I B$_1$	0.328	0.036	0.014	0.010		0.388

Human type Fraction 520 C precipitated 0.780 mg. of N.
* 0.2 mg. of polysaccharide used.

the C, B$_{2a}$, and B$_1$ fractions of the human strain (3) were also used in absorption studies on pooled anti-avian rabbit serum.

DISCUSSION

The amount of polysaccharides isolated from defatted cells of Strain 531 of the avian tubercle bacillus was only about one-half that obtained from similar quantities of the human and avian strains studied. While this may not be a constant characteristic of the three types of tubercle bacilli, a similar variation did not occur in the amount of water-soluble polysaccharides carried over into the lipids of these strains, as the quantities found were approximately equal (7).

On the basis of the quantities of serologically active and inactive carbohydrate fractions isolated from the avian bacillus this strain appears to occupy an intermediate position between the human and bovine strains. Lot 555 contained 79 per cent of material serologically active at 1:500,000 dilution, while Lot 554 contained 70 per cent. It will be recalled (cf. (4) Table V) that 87 per cent of the polysaccharides isolated from defatted cells of the H-37 human strain of tubercle bacillus was serologically active at a dilution of 1:500,000, while only 17 per cent of active fractions could be recovered from cells of the bovine type. Moreover, in the case of the bovine polysaccharides it was found advantageous to work up the percolate in two successive portions, as the serologically inactive polysaccharides were concentrated to a considerable extent in the earlier portions of the extract ((4) Table V). The corresponding avian percolates, as shown by the fractionation of Lots 554 and 555, contained almost the same proportion of active and inert components.

The ease with which the avian polysaccharides passed through cellophane into water was at first surprising, but it was found that even the human type fraction, 520 C, which contained combined magnesium palmitate (3), dialyzed with equal ease. It is therefore probable that the tubercle bacillus polysaccharides do not have the high molecular weights characteristic of many of the bacterial polysaccharides in undegraded form, and this is further indicated by the failure of their aqueous solutions to show high viscosities.

The qualitative and quantitative serological tests show that the avian fractions exhibit immunological specificities similar to those of the corresponding fractions of the human and bovine types (see also Table VI). The independent specificity of certain of the B fractions is equally marked. The I C_1 fraction corresponds chemically with the C fraction of the human strain in that both split off magnesium palmitate when treated with alkali, but the avian I C_1 precipitated far less antibody from both anti-H-37 horse serum and anti-avian rabbit serum than did the human type C fraction ((3) and Tables III and V). Evidence of an independent C specificity, as in the human strain, was obtained, however, since Serum 5807-L, absorbed with avian Fraction 554 I B_1, gave precipitates with Fractions 554 I C_1 and I C_{2a}. Many

TABLE VI

Qualitative Tests of Carbohydrate Fractions in Absorbed Antisera

Fraction used for testing absorbed sera	Anti-human type horse serum No. 5807-L absorbed with					Anti-avian type rabbit serum pool absorbed with				
	554 I C1	554 I C3a	554 I C3b	554 I B1	554 I C1	554 I C3a	554 I C3b	520 C	520 B3a	520 B1
554 I C1	− (+)	− (−)	− (−)	− (+)	− (−)	− (−)	− (+?)	+ (+±)	− (±)	
I C2a	+± (+++±)	− (−)	− (−)	− (+)	+± (+++±)	− (−)	− (−)	± −	− −	++ (+++±)
I C2b	+± (+++)	− (−)	− (−)	− (+)	+± (+++±)	− (−)	− (−)			+ (+++)
I C4	++ (+++±)	− (−)	− (−)	− (+)	+± (+++)	− (−)	− (−)			+± (+++)
I B2	++ (+++±)	+(+)	− (−)	± (±)	++ (+++)	+± −	+± −			± (+++)
I B1	++ (+++±)	− (+±)	− (−)	− (−)	++ (+++)	+± −	− −	± −	± −	− −
520 C	− (−)	− (+±)	− (+)	± (+±)	+± (+++)	− (−)	− −	± −	− −	+ (+++)
B2a	++ (+++)	− (+)	− (−)	− (+?)	+ (+++)	− (−)	− −	− −	− −	+ (+++)
531 C1	++ (++)	− (−)	− (−)	− (+±)	+± (+++)	− (−)	± −	± −	+? −	+± (+++)
B2	++ (+++)	+ (+++±)	− (−)	− (−)	+ (+++)	− (−)	− −	− −	− −	± (+)
550 I C1*								± −	− −	+± (+++)
I B2*								± −	− −	− −
I B1*								± −	− −	− −

Fractions I C4 and I B2 removed all antibodies from Serum 5807-L, and Fractions I C4, I B2, and I B1 behaved similarly in the rabbit serum.

* From an earlier preparation, not described in the present paper.

of the avian fractions (Nos. 554 I C_6, I B_2, and I B_4) also resembled corresponding products from the human type in removing all precipitins from both anti-H-37 and anti-avian type sera, thus showing a combination of B and C specificities in spite of rigorous fractionation. Others, such as Fractions 554 I B_1, I C_{2a}, and I C_{2b}, failed to remove all precipitins from the anti-H-37 horse serum but absorbed them from the anti-avian rabbit serum. In spite of this no claim can be made that any fraction was avian-specific. Absorption of anti-avian rabbit sera with the human strain Fraction 520 B_{2a} completely removed precipitins for all avian strain polysaccharides, and, conversely, absorption of the sera with the avian strain Fraction 554 I B_2 removed precipitins for the principal human strain polysaccharides which were tested.

In the quantitative absorptions of Serum 5807-L the I B_2 fractions yielded the largest amounts of precipitable nitrogen, but in the pooled anti-avian rabbit sera Fractions 554 I C_{2a} and I C_{2b} removed more antibody nitrogen than did the others. However, even slightly more antibody nitrogen was removed from this serum by the human type Fraction 520 C, although the absorbed serum still contained antibodies precipitable by Fraction 550 B_2^2 and by the human type Fractions 520 B_{2a} and 520 B_1 (see also Table VI).

As in the case of the human and bovine type fractions, the avian type polysaccharides high in serological activity were also generally found to be high in pentose, lending further support to the suggestion (4, 8) that the active fractions contain pentose as an essential component. Serologically inert carbohydrate fractions high in organic phosphorus seemed lacking in the avian strain, although products of this nature were isolated from cells of both the human and bovine types. The more soluble phosphorylated but serologically inactive carbohydrate in Fractions 530 C_{4a} and C_{4b}, which seemed specific for the bovine strain (4), was not encountered, so that this product would still seem to be a chemical substance characteristic of the bovine strain. Other differences were also noted. For example, fractions corresponding to the serologically inert human type Fractions 520 B_1C and B_2C

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[2] From an earlier preparation, not described in the present paper.

and the bovine Fractions 530 B_1C, B_2C, and B_3C were not encountered. Instead, appreciable amounts of magnesium sulfate separated under conditions in which these fractions were expected. In this sense an inorganic salt might be said to be the only avian-specific substance isolated in the course of the present study.

SUMMARY

1. The polysaccharides extracted from the cells of an avian strain, No. 531, of tubercle bacillus were intermediate between those isolated from human and bovine strains with respect to the proportion of serologically active material, but the yield of polysaccharides was lower than in the other types.

2. The specific polysaccharides showed the same immunological specificities as did those of the human and bovine types. The portion corresponding to the C fraction in these types was weaker in serological activity.

3. No soluble serologically inactive, phosphorylated carbohydrate corresponding to that found in the bovine strain was encountered.

4. Serological activity of the avian polysaccharide is highest in the fractions containing pentose, as in the human and bovine types.

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THE URINARY EXCRETION OF STEROID COMPOUNDS

I. NORMAL MALE SUBJECTS*

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This study represents an attempt to develop a procedure for the separation and isolation of steroid compounds from the urine of normal male subjects.

Methods

A concentrate of 1000 liters of human male urine was prepared by the method of Curtis, MacCorquodale, Thayer, and Doisy (1). After removal of acidic and phenolic material, the neutral fraction of this concentrate was treated with Girard's Reagent P (2). From the ketonic and non-ketonic fractions thus obtained, alcoholic constituents were removed as half succinates (3). The alcoholic fractions were treated with an excess of digitonin in order to separate the C_3 epimers.

From the six fractions obtained, five crystalline compounds were isolated and identified: *trans*-dehydroandrosterone (68 mg.), androsterone (117 mg.), etiocholanol-3(α)-one-17 (27 mg.), cholesterol (59 mg.), and pregnanediol-3(α),20(α) (63 mg.).

Further investigations of the syrupy portions of the non-ketonic alcoholic fractions as well as the non-alcoholic ketonic fraction and the non-ketonic non-alcoholic fraction will be reported at a later date.

DISCUSSION

It must be pointed out that the yields of the known compounds, *trans*-dehydroandrosterone, androsterone, etiocholanol-3(α)-one-17, and cholesterol (Table I) are considerably lower than those reported by other workers. While no figures are available for the amount of pregnanediol-3(α),20(α) in human male urine, the same considerations apply. We feel that this discrepancy can be attributed to the relative inefficiency of the benzoic acid adsorption method as compared with the solvent extraction procedures used by other workers and that the actual and relative

TABLE I

Steroid Compounds in Human Male Urine

Compound	Quantity isolated	
	Present authors*	Previous authors†
	mg. per l.	*mg. per l.*
trans-Dehydroandrosterone..... . .	0.07	0.2(4)
Androsterone...............	0 17	1.6(4)
Etiocholanol-3(α)-one-17...	0.03	1.4(4)
Cholesterol.............	0.06	0.01‡(5)
		0.3 §(6, 7)
Pregnanediol-3(α),20(α)................	0.06	

* The amounts of pure substances isolated have no quantitative physiological significance.

† The figures in parentheses represent bibliographic references.

‡ Based on the amount of analytically pure cholesteryl acetate isolated.

§ This value was obtained by the extraction of unhydrolyzed urine.

amounts of pure substances isolated by us have no quantitative physiological significance.

In a recent publication Callow and Callow (4) have suggested that since *trans*-dehydroandrosterone is excreted in increased amounts (2 mg. per liter) in the urine of a eunuch, this compound may be of extragonadal (adrenal cortex?) origin. This is consistent with the observation that the normal female (8) as well as the castrate female (9) excretes this compound in an amount comparable with that excreted by the normal male (10). Additional evidence bearing on this point is provided by the observation of Crooke and Callow (11) that there is an increased excretion of

trans-dehydroandrosterone in patients with adrenal cortical tumors.

Androsterone and etiocholanol-3(α)-one-17 are also constituents of the urine of normal males (10), castrate males (4), normal females (8), and castrate females (9), although the amounts excreted by the castrate male are significantly smaller (0.6 mg. of androsterone and 0.9 mg. of etiocholanol-3(α)-one-17 per liter) than those excreted by the normal male (Table I). In addition, Marker and Lawson (12) have isolated androsterone from the urine of pregnant women and Butler and Marrian (13, 14) have obtained etiocholanol-3(α)-one-17 from the urine of a woman suffering from adrenal hyperplasia. The observation of Callow (10) and Dorfman, Cook, and Hamilton (15) that the injection of massive doses of testosterone in male patients suffering from hypogonadism is followed by an increased excretion of androsterone and etiocholanol-3(α)-one-17 suggests that in part, at least, these compounds are of gonadal origin and indicates that the reduction of testosterone can take place in some site other than the testis.

Further evidence on this point has been obtained by Dorfman and Hamilton (16) who observed an increased amount of androsterone in the urine of a eunuchoid patient following the oral administration of testosterone, androsterone, androstanediol-3(α),17, androstanedione-3,17, and androstene-4-dione-3,17.

Pregnanediol-3(α),20(α) is generally recognized as a normal constituent of gravid and non-gravid human female urine. The isolation of pregnanediol-3(α),20(α) from human male urine and from the urine of a normal male subject following the injection of progesterone (17) suggests that the human male is able to effect the reduction of progesterone to pregnanediol. Marker, Wittle, and Lawson (18) have isolated pregnanediol-3(α),20(α), allo-pregnanediol-3(α),20(α), and allopregnanediol-3(β),20(α) from bull urine but no traces of the pregnanediols could be obtained from steer urine (19). Beall (20) was able to isolate progesterone and allopregnanol-3(β)-one-20 from ox (steer) adrenal glands. This would suggest that in this species the testis may play some rôle in the reduction of progesterone to the pregnanediols and that in the absence of the testis the degradation proceeds to unrecognizable products. In this connection it is interesting to note that

no pregnanediols could be recovered from the urine of the pregnant rhesus monkey following the injection of progesterone or pregnanediol (21).

EXPERIMENTAL

Collection and Hydrolysis—Normal human male urine was collected in 5 gallon carboys and preserved under toluene. To each 10 liter portion of urine after removal of the toluene, 1 liter of concentrated hydrochloric acid was added and the mixture was allowed to stand at room temperature for 7 to 10 days.

Precipitation—The hydrolysate was then precipitated with sodium benzoate according to the procedure described by Curtis, MacCorquodale, Thayer, and Doisy (1) with 20 gm. of sodium benzoate per liter of urine. 20 kilos of benzoic acid were obtained from 1000 liters of urine.

Preparation of Benzoic Acid-Free Concentrate—(A) 200 gm. of benzoic acid were shaken vigorously with 1500 ml. of dry ether which had previously been used in step (B) and the clear ether solution filtered off. (B) The residue was then extracted with four 400 ml. portions of fresh ether and the insoluble material filtered off and discarded. (C) The filtrate from (A) was shaken with sodium hydroxide until the aqueous solution was just alkaline to phenolphthalein. Decreasing volumes of alkali were used so that the last washings were carried out with 25 ml. portions. (D) The sodium hydroxide washings were combined and shaken out with four 400 ml. portions of ether. The ether washings were combined with the alkali-washed ether solutions, allowed to stand overnight in a dry vessel, and evaporated to a syrup on the steam bath. (E) The syrups obtained from several batches of benzoic acid were combined, taken up in ether, and washed with sodium hydroxide until the washings were alkaline to phenolphthalein. The washings were shaken back with ether and the combined ether solutions were concentrated to a syrup on the steam bath. The final product was a viscous dark red syrup.

Separation of Neutral and Acidic Fractions—The crude syrup from 1000 liters of urine was dissolved in 1 liter of 10 per cent ethanolic potassium hydroxide and boiled under a reflux for 3 hours. The reaction mixture was then evaporated to about 500 ml. and poured into a mixture of 1 kilo of ice, 1000 ml. of water,

and 550 ml. of 5 N hydrochloric acid and shaken out with butanol (3 liters). The butanol solution was washed with nineteen 100 ml. portions of 5 per cent sodium bicarbonate. The bicarbonate washings were shaken back once with butanol.

The butanol solution was diluted with 2 volumes of petroleum ether (b. p. 35–60°) and shaken out with 100 ml. portions of 2 N sodium hydroxide. The alkaline washings were shaken back three times with a butanol-petroleum ether mixture. The butanol-petroleum ether solution was then washed with 100 ml. portions of water until the washings were colorless. The combined alkali and water washings were acidified to Congo red with 5 N hydrochloric acid and extracted with ether. The ether solution was washed with water, dried over sodium sulfate, and evaporated to dryness, yielding 1.52 gm. of acidic and phenolic material.

The butanol-petroleum ether solution was washed with 100 ml. portions of 2 N hydrochloric acid until the washings were colorless, and finally with water. After removal of the solvents 18.3 gm. of neutral material were obtained.

Separation of Neutral Ketones from Neutral Non-Ketonic Fraction—The neutral fraction (18.3 gm.) was dissolved in 120 ml. of absolute ethanol and boiled under a reflux for an hour with 12 gm. of glacial acetic acid and 6.8 gm. of Girard's Reagent P (2). After the mixture was worked up in the usual manner, 3.81 gm. of neutral ketonic and 9.98 gm. of neutral non-ketonic material were obtained.

Separation of Alcoholic from Non-Alcoholic Neutral Ketones—The neutral ketonic fraction (3.81 gm.) was dissolved in 60 ml. of dry pyridine and treated with 12 gm. of succinic anhydride (3). After being warmed gently to effect solution the reaction mixture was allowed to stand at room temperature for 46 hours, warmed on the steam bath for $\frac{1}{2}$ hour, and poured into 750 ml. of ice water. By the usual methods, 1.03 gm. of neutral non-alcoholic ketonic and 2.32 gm. of neutral alcoholic ketonic material were isolated.

Separation of Alcohols from Neutral Non-Ketonic Fraction—The neutral non-ketonic fraction (9.98 gm.) was treated with 30 gm. of succinic anhydride and 150 ml. of pyridine. 3.93 gm. of neutral non-ketonic non-alcoholic material and 4.29 gm. of neutral alcoholic material were obtained.

All four fractions were retreated with Girard's Reagent P. The neutral alcoholic ketone fraction (2.32 gm.) yielded 0.38 gm. of neutral non-ketonic alcohols and 1.85 gm. of neutral ketonic alcohols; the neutral non-alcoholic ketone fraction (1.03 gm.) yielded 0.22 gm. of neutral non-ketonic non-alcoholic material and 0.77 gm. of neutral non-alcoholic ketones; the neutral non-ketonic alcohol fraction (4.29 gm.) yielded 3.98 gm. of neutral non-ketonic alcohols and 0.21 gm. of neutral ketonic alcohols; and the neutral non-ketonic non-alcoholic fraction (3.93 gm.) yielded 0.06 gm. of neutral ketonic non-alcoholic material and approximately 3.75 gm. of neutral non-ketonic non-alcoholic material which was not weighed because of the difficulty in drying the material to constant weight.

After combination of similar fractions the four fractions were retreated with succinic anhydride and pyridine. The neutral non-ketonic alcohol fraction (4.36 gm.) yielded 0.66 gm. of neutral non-ketonic non-alcoholic material and 3.41 gm. of neutral non-ketonic alcohols; the neutral ketonic alcohol fraction (2.06 gm.) yielded 0.54 gm. of neutral non-alcoholic ketones and 1.42 gm. of neutral alcoholic ketones; the neutral non-ketonic non-alcoholic fraction (about 3.9 gm.) yielded about 3.1 gm. of neutral non-alcoholic non-ketonic material and 0.34 gm. of neutral non-ketonic alcohols; and the neutral non-alcoholic ketone fraction (0.83 gm.) yielded 0.44 gm. of neutral non-alcoholic ketones and 0.29 gm. of neutral ketonic alcohols.

Digitonin Precipitation of Neutral Ketonic Alcohols—The neutral ketonic alcohol fractions were combined (1.71 gm.) and dissolved in 200 ml. of hot 60 per cent methanol. The clear solution was treated with a hot solution of 4 gm. of digitonin in 100 ml. of hot 60 per cent methanol. Precipitation of digitonides took place immediately. After the material was chilled overnight the precipitate was filtered off, washed with a small quantity of ice-cold 60 per cent methanol, and dried.

The filtrate and washings were evaporated to dryness under reduced pressure. The residue was dissolved in absolute ethanol and again taken down to dryness under reduced pressure. The final residue was warmed on the steam bath for $\frac{1}{2}$ hour with 15 ml. of dry pyridine. The solution was poured into 150 ml. of dry ether and the precipitated digitonin was filtered off and washed with ether. The filtrate and washings were washed with

N hydrochloric acid and then with water. The solution was dried over sodium sulfate and evaporated to dryness. The yield of neutral ketonic alcohols not precipitated by digitonin was 1.38 gm.

The solid digitonides were heated on the steam bath for $\frac{1}{2}$ hour with 10 ml. of pyridine and the solution was poured into 100 ml. of ether. The precipitated digitonin was filtered off and washed with ether. The filtrate and washings were washed with N hydrochloric acid and water, dried over sodium sulfate, and evaporated to dryness. 0.38 gm. of neutral digitonin-precipitable ketonic alcohols was obtained.

Digitonin Precipitation of Neutral Non-Ketonic Alcohols—The neutral non-ketonic alcohol fraction (3.76 gm.) was dissolved in 500 ml. of hot 60 per cent methanol and treated with a hot solution of 8.0 gm. of digitonin in 200 ml. of hot 60 per cent methanol. The mixture was chilled overnight and the digitonides filtered off, washed with cold 60 per cent methanol, and dried.

The precipitate and filtrate were worked up as above and yielded respectively 1.73 gm. of neutral non-ketonic digitonin-precipitable alcohols and 1.97 gm. of neutral non-ketonic alcohols not precipitated by digitonin.

Isolation of Trans-Dehydroandrosterone—The neutral ketonic digitonin-precipitable alcohol fraction (0.38 gm.) was extracted repeatedly with boiling hexane. The hexane extracts were filtered and allowed to evaporate slowly at room temperature. The fine colorless needles which separated were filtered off and recrystallized once from hexane. 38 mg. of material, m.p. 145–146.5°,[1] were obtained. The mother liquors were evaporated to dryness and the residue was treated with 1 ml. of pyridine and 0.3 ml. of benzoyl chloride and heated on the steam bath for 15 minutes. The solution was diluted with water and filtered. The crystals were washed with cold methanol and recrystallized from ethyl acetate. *trans*-Dehydroandrosterone benzoate (15 mg.) melting at 229–235° was obtained.

The material insoluble in hexane was dissolved in 10 ml. of methanol and boiled under a reflux with 300 mg. of semicarbazide hydrochloride and 350 mg. of sodium acetate (hydrated) for 2 hours. After the material was chilled, the precipitate was filtered

[1] All the melting points are corrected.

off and recrystallized from 95 per cent ethanol, yielding 36 mg. of *trans*-dehydroandrosterone semicarbazone, m.p. 263–265°.

The benzoate was also prepared from 36 mg. of the free ketone, m.p. 146–146.5°, and 13 mg. of benzoate, m.p. 247–250°, and 10 mg. of material, m.p. 241–244°, were obtained. When the higher melting fraction was mixed with an authentic specimen of *trans*-dehydroandrosterone benzoate, no depression of the melting point was observed.

Isolation of Androsterone and Etiocholanol-3(α)-One-17—The fraction consisting of neutral ketonic alcohols not precipitated by digitonin (1.38 gm.) was extracted repeatedly with boiling hexane and the extracts evaporated to dryness. The residue was acetylated by treatment with 3 ml. of acetic anhydride and 3 ml. of pyridine on the steam bath for 2½ hours. The reaction mixture was diluted with water and chilled. The precipitated solid was filtered off and washed with water.

The brown hexane-insoluble residue was dissolved in 20 ml. of methanol and treated with a solution of 0.5 gm. of semicarbazide hydrochloride and 0.6 gm. of sodium acetate. After boiling under a reflux for 2 hours the mixture was cooled, filtered, and the semicarbazone washed with water and methanol and dried. The semicarbazone mixture was suspended in a mixture of 7.5 ml. of ethanol, 4 ml. of water, and 1 ml. of concentrated sulfuric acid and refluxed for 2 hours. The reaction mixture was diluted with water and extracted with ether. The extract was washed with water, dried over sodium sulfate, and evaporated to dryness. The residue was acetylated by treatment with 1 ml. of acetic anhydride and 1 ml. of pyridine on the steam bath for 2 hours. The reaction mixture was diluted with water and chilled, and the partly crystalline solid filtered off, washed with water, and combined with the main batch of acetates.

The crude acetates were fractionally crystallized from aqueous methanol by the "triangle method." From the least soluble fractions 134 mg. of androsterone acetate, m.p. 159.5–161.5°, were obtained. Saponification yielded androsterone, m.p. 182–183°, after one recrystallization from aqueous methanol. The melting point was not depressed upon admixture with an authentic specimen of androsterone.[2]

The more soluble material yielded fractions ranging in melting point from 84–113°. These were combined and boiled under a reflux for $1\frac{1}{2}$ hours with 10 ml. of 5 per cent methanolic potassium hydroxide. After standing at room temperature overnight, the reaction mixture was diluted with water, extracted with ether, and the extract washed with water, dried over sodium sulfate, and evaporated to dryness.

The pale yellow syrupy residue was dissolved in 35 ml. of carbon tetrachloride and passed through a column of aluminum oxide (Merck, standardized according to Brockmann) 15 mm. in diameter and 150 mm. in height. The column was developed with 100 ml. of carbon tetrachloride and then eluted with 100 ml. portions of 0.1, 0.2, 0.3, and 0.5 per cent ethanol in carbon tetrachloride and finally with 200 ml. of 1 per cent ethanol in carbon tetrachloride.

The fraction eluted by 0.5 per cent ethanol in carbon tetrachloride was dissolved in 0.5 ml. of pyridine, treated with 0.05 ml. of benzoyl chloride, and warmed on the steam bath for 15 minutes. After dilution with water the mixture was chilled overnight. The water was decanted off and the residual oil washed with water and taken up in methanol. After dilution with water and chilling, crystals, m.p. 151–154.5°, separated. One recrystallization raised the melting point to 162–163°. The melting point was not depressed by admixture with an authentic specimen of etiocholanol-3(α)-one-17.[3] 37 mg. of etiocholanol-3(α)-one-17 benzoate were obtained. Saponification with methanolic sodium hydroxide yielded etiocholanol-3(α)-one-17, m.p. 150–151°, after one recrystallization from aqueous methanol. The melting point was not depressed by admixture with an authentic specimen of etiocholanol-3(α)-one-17.[3]

Isolation of Cholesteryl Acetate—The neutral non-ketonic digitonin-precipitable alcohol fraction (1.73 gm.) was dissolved in 7 ml. of pyridine and 5 ml. of acetic anhydride, warmed on the steam bath for $\frac{1}{2}$ hour, and allowed to stand at room temperature for 22 hours. After evaporation to dryness under reduced pressure, the residue was dissolved in 100 ml. of ether and the solution was washed with dilute hydrochloric acid, dilute sodium carbonate, and water, dried over sodium sulfate, and evaporated to dryness.

[3] The authors are indebted to Dr. H. Hirschmann for the specimen of etiocholanol-3(α)-one-17 and its benzoate.

The residual syrup was dissolved in hot 95 per cent ethanol, filtered, and chilled. The semicrystalline precipitate was filtered off and, after three recrystallizations from ethanol and one from ether-ethanol mixture, 65 mg. of cholesteryl acetate, m.p. 114–115°, were obtained. The melting point was not depressed on admixture with an authentic specimen of cholesteryl acetate.

Isolation of Pregnanediol-3(α),20(α)—The neutral non-ketonic alcohols not precipitated by digitonin (1.97 gm.) were dissolved in 8 ml. of pyridine and 6 ml. of acetic anhydride, heated on the steam bath for $1\frac{1}{2}$ hours, and allowed to stand at room temperature for 22 hours. The reaction mixture was evaporated to dryness under reduced pressure and the residue dissolved in ether. The ether solution was washed with dilute hydrochloric acid, dilute sodium carbonate, and water, dried over sodium sulfate, and evaporated to dryness.

The residue was dissolved in 10 ml. of benzene and poured through a column of aluminum oxide 420 mm. in height and 8 mm. in diameter. The column was then washed with successive portions of pentane (four 10 ml. washings), benzene (five 10 ml. washings), ethyl acetate (four 10 ml. washings), acetone (two 10 ml. washings), and methanol (two 10 ml. washings). The first two pentane washings were evaporated to dryness and the residual clear yellow syrups were dissolved in cold methanol and diluted with a small amount of water. After the material was·chilled, the semicrystalline products were filtered off and combined.

After two recrystallizations from methanol 73 mg. of pregnanediol-3(α),20(α) diacetate, m.p. 176.5–178°, were obtained. From the mother liquors an additional 6 mg., m.p. 176.5–178.5°, were isolated. No depression of the melting point was observed when this compound was mixed with an authentic specimen of pregnanediol-3(α),20(α) diacetate.

Saponification of the diacetate by boiling for 2 hours with 10 per cent methanolic potassium hydroxide and recrystallization of the product from aqueous ethanol yielded pregnanediol-3(α),-20(α), m.p. 234.5–236.5°, which was not depressed by admixture with an authentic specimen.

SUMMARY

1. A systematic procedure for the separation and isolation of steroid compounds from urine has been developed and applied to the urine of human male subjects.

2. The isolation and identification of androsterone, etiocholanol-3(α)-one-17, *trans*-dehydroandrosterone, cholesterol, and pregnanediol-3(α),20(α) are described.

3. Theories concerning the origin of these compounds are discussed.

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INCREASED EXCRETION OF URINARY AMMONIA IN THE DOG FOLLOWING THE INTRAVENOUS INJECTION OF BOTH NATURAL AND UNNATURAL FORMS OF CERTAIN AMINO ACIDS*

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The purpose of this investigation was to determine whether or not there would be an increased excretion of urinary ammonia when amino acids were furnished as precursors for this ammonia formation in addition to the amino acids already available in the blood stream. When it was found that there was increased ammonia elimination following the intravenous injection of amino acids, attention was directed to the effectiveness of the natural and unnatural forms.

Until recently the view has been held that tissue proteins yield only the l forms (Wohl-Freudenberg nomenclature) of amino acids on hydrolysis, and, under the circumstances, only these natural forms were thought of as being available for any reactions that amino acids might undergo in the animal body. This view was fortified by a large body of evidence which has been reviewed by Berg and Potgieter (1). They state, "It is obvious, therefore, that the unnatural component of a racemic modification of an amino acid is less readily attacked in the body than is the naturally occurring isomer." These authors raised the question whether this difference in utilization is an absolute one or not. "Whether this difference in susceptibility to attack is sufficient to render effective utilization of an optical isomer impossible when only small amounts of its antipode are required (as, for example, in growth) may well be questioned."

Evidence has been accumulating to indicate that the animal

body can utilize at least some of the unnatural isomers of the amino acids. In 1937 Conrad and Berg (2) demonstrated the transformation of the unnatural *d*-histidine into *l*-histidine in the growing rat. These studies were extended to tryptophane and lysine by Totter and Berg (3), showing that, like *d*-histidine, *d*-tryptophane can be utilized, whereas *d*-lysine is non-utilizable.

Kögl and Erxleben (4) claim to have demonstrated that the unnatural (*d*) form of glutamic acid can be isolated from cancer tissue, but not from normal tissue. In the controversy raised by this claim, evidence has been offered (5) to indicate that the un-natural form of glutamic acid is present in various normal proteins, thus requiring a reexamination of the view generally held that proteins contain only the natural (*l*) optical antipode of the amino acids.

The work of Braunstein and Kritsman (6) and Braunstein (7) on the transamination of *d* and *l* forms of the amino acids in normal muscle and malignant tissue adds further interest to the question of the biological importance of the natural and unnatural antipodes of the amino acids.

It has been established that amino acids are deaminated, with ammonia formation, when incubated with fresh kidney slices (8, 9). In the present investigation we have attempted to demonstrate that this occurs in the living animal.

The work of Polonovski and Boulanger is related to this problem. These investigators (10) studied kidney arterio-venous differences in ammonia content, and they concluded that increased differences in ammonia content were obtained when glycine, *dl*-alanine, and *l*-alanine (slight) were given intravenously, whereas the injection of urea was without effect upon such differences. They report no studies on urine, but conclude that, "These amino acids appear to contribute to the production of ammonia in the urine." In further studies (11) these authors again measured only the blood ammonia differences between renal artery and vein. They studied the effects of natural and unnatural forms of alanine by injecting the *dl* form and the *l*-alanine (referred to by them as "*d*-alanine (*alanine naturelle*)"), observing that more ammonia appeared in the renal vein when the *dl* form was used than when *l*-alanine was used.

Our approach to the problem has been to attempt to demon-

strate that amino acids do or do not serve as precursors for the urinary ammonia formed in the kidney for acid neutralization. We have reasoned that if amino acids do serve as precursors for the urinary ammonia their intravenous injection should augment ammonia formation when the kidney is stimulated to ammonia formation by the administration of mineral acid.

Methods

Except where specified to the contrary, all experiments were performed with unanesthetized dogs that had been trained to metabolic procedures. Females were used. They were maintained on a diet of 70 calories per kilo, in which lean beef muscle furnished 0.61 gm. of nitrogen per kilo, and the remainder of the calories was furnished as sucrose. On this diet dogs maintain a constant weight and remain in vigorous condition for considerable periods.

In a preliminary operation the vagina is split to facilitate catheterization. To insure accurate collection of urine in the 75 minute periods, the bladder was always washed out with measured portions of sterile water, which was completely recovered in all cases, and such washings were continued and added to the urine sample until the washings contained no urinary pigment. Analyses were made at once in duplicate.

Dogs were always fed at 5 p.m. and experiments started at 9 a.m., so that in each case the experiment started 16 hours after the feeding of a constant standard diet, thus standardizing the time of the experiment in the last 8 hours before a subsequent feeding. In all cases the food was eaten immediately and completely.

Ammonia was determined by aeration of a 5 cc. sample into 0.1 N acid, followed by titration with base. Caprylic alcohol was used as an antifoam, and Folin's alkali mixture of 15 per cent potassium carbonate and 15 per cent potassium oxalate was used, at least 1 cc. of excess being allowed after the urine had been rendered alkaline. Control experiments showed that recovery was complete in 30 minutes; the aeration periods were 45 minutes.

Amino acids were determined by Danielson's (12) modification of the Folin (13) method.

Urea was estimated by the urease method given in Peters and

Van Slyke (14) with the following modifications: The urease preparation used was 50 mg. of urease, Squibb, powdered. Urea nitrogen was calculated by subtracting the blank on the reagents as well as the preformed ammonia determined separately by aeration as described above.

The amino acids used were obtained from Hoffmann-La Roche, Inc. Their purity and identity were established by determination of total nitrogen, amino nitrogen, and rotation in HCl solution.

We have compared the ammonia formation after acid administration in a preliminary period in which there was acid administration only with the ammonia formation after acid administration plus the intravenous injection of amino acids which could serve as additional precursors of urinary ammonia at a time when the kidneys are called upon to produce additional ammonia, and with an after period when again, as in the preliminary period, there was acid administration only. 150 cc. of 0.14 N HCl were given by stomach tube in each case.

The experiments were carried out in the following way: 16 hours after the last feeding the bladder was emptied and washed with sterile water. 100 cc. of water were given by stomach tube to insure adequate urine volumes. Two preliminary, 75 minute urine samples were collected. 150 cc. of 0.14 N HCl were given by stomach tube, and the urine collected for three more 75 minute periods. When amino acids were given in addition to mineral acid, the injections into the jugular vein were accomplished as follows: 2 gm. of the amino acid were dissolved in water to a volume of 100 cc. This solution at body temperature was injected in five portions of 20 cc. each at about equal intervals during the 1st hour after the mineral acid had been given by stomach tube. No anesthesia was used, and all urines were direct catheter specimens, although the dogs were kept in metabolism cages between catheterizations to guard against the possibility of spontaneous urinations. By washing out the bladder at the end of each catheterization, we were convinced that complete emptying was achieved, and, after some experience had been acquired, it was possible to obtain emptyings at the exact intervals within a few seconds.

Table I is compiled from the data on three dogs and shows that comparable values for the preliminary and after periods of acid

administration alone are obtainable, whereas the administration of amino acid at the time of imposing this acid load causes an output of urinary ammonia that is 2 to 4 times the amount eliminated after acid alone.

Table II presents results of controls on acid administration, showing that there is always an increase in urinary ammonia following acid administration, and that the amount of such *extra*

TABLE I

Rate of Ammonia Elimination in Urine in Response to Acid Administration and Injection of Amino Acids

150 cc. of 0.14 N HCl were administered by stomach tube. The amino acids were injected into the jugular vein.

Dog No.	Date	Period*	Extra† ammonia N excreted
	1939		*mg. per hr.*
1	Feb. 14	Preliminary	6.2
	" 22	Experimental	15.2 (1.8 gm. *dl*-leucine)
	Mar. 9	After	6.4
2	" 13	Preliminary	4.2
	" 15	Experimental	19.8 (2.0 gm. *d*-alanine‡)
	Apr. 10	After	4.0
3	Mar. 6	Preliminary	7.9
	" 13	"	6.4
	" 16	Experimental	14.8 (2.0 gm. *d*-alanine‡)
	" 21	After	7.5
	" 30	"	7.9

* Preliminary period, acid administration only; experimental, acid administration and injection of amino acid; after period, acid administration only.

† The difference between fore and after periods on a single day's experiment.

‡ Unnatural isomer.

ammonia nitrogen elimination is well below the *extra* amount eliminated when amino acids are given.

When urea is injected in addition to acid administration (with more than double the amount of nitrogen used in the amino acid experiments), there is no more ammonia eliminated than when acid is administered alone. Also, the injection of amino acid alone (in the largest amount used in any experiment) causes no such ammonia elimination as was obtained when acid was given,

although the small augmentation of ammonia elimination is in harmony with the view that amino acids are the precursors of urinary ammonia.

From the data in Table III it is seen that the injection of both optical enantiomorphs of alanine and leucine causes the elimination

TABLE II

Excretion of Urinary Ammonia Nitrogen before and after HCl by Stomach Tube; Controls on Acid Administration Alone

Each reading represents the average of two or more 75 minute periods.

Dog No.	Preliminary	After HCl by stomach tube	Net increase
	mg. per hr.	*mg. per hr.*	*mg. per hr.*
1	8.6	14.7	6.1
4	4.1	10.9	6.8
2	7.0	16.7	9.7
3	7.9	16.2	8.3
2	8.1	18.4	10.3
1	16.8	23.0	6.2
2	7.5	12.0	4.5
3	7.9	14.4	6.5
3	11.7	20.0	8.3
3	10.6	18.0	7.4
4	7.6	14.1	6.5
5	8.8	17.3	8.5
6	6.5	9.8	3.3
6	15.0	19.6	4.6
7	12.1	15.9	3.8
7	12.3	19.1	6.8
6	10.0	16.2	6.2
Average............ 9.6		16.3	6.7

Control, urea injection (1.5 gm.) + acid administration			
2	10.8	17.7	6.9

Control, 6.0 gm. *dl*-alanine injected without acid administration			
5	13.1	19.0	5.9

of more *extra* ammonia following the administration of hydrochloric acid than was eliminated when hydrochloric acid was given alone. Also, in all cases there is somewhat more ammonia elimination following the injection of the unnatural isomer than with the natural one.

TABLE III

Excretion of Urinary Ammonia Nitrogen before and after HCl by Stomach Tube and Amino Acid Intravenously

Each figure represents the average of two or more 75 minute periods.

Amino acid	Prelimi-nary	After HCl and amino acid	Net in-crease	Amino acid	Prelimi-nary	After HCl and amino acid	Net in-crease
	mg. per hr.	*mg. per hr.*	*mg. per hr.*		*mg. per hr.*	*mg. per hr.*	*mg. per hr.*
dl-Alanine	7.8	23.7		*dl*-Leucine	9.8	24.8	
	9.1	23.5			2.7	10.5	
	(8.5)	(23.6)	15.1		(6.3)	(17.7)	11.4
l-Alanine	6.0	15.6		*l*-Leucine	9.9	21.9	
	7.9	17.1			20.4	29.9	
	8.7	26.2			(15.2)	(25.9)	10.7
	(7.5)	(19.6)	12.1	*d*-Leucine	9.3	25.2	
d-Alanine	6.3	25.2			10.4	21.0	
	12.8	28.0			(9.9)	(23.1)	13.2
	13.3	30.6					
	7.8	*19.7*					
	(10.1)	(25.9)	15.8				

The figures in parentheses represent the averages for the groups.

TABLE IV

Excretion of Ammonia, Amino Acid, and Urea Nitrogen

The amounts shown represent the extra elimination following the administration of HCl (by stomach tube) and amino acid (intravenously).

	Dog No.	Ammonia N	Amino acid N		Urea N
				Total excreted	
		mg. per hr.	*mg. per hr.*	*mg.*	*mg. per hr.*
Natural forms					
l-Leucine (2.0 gm. containing 213.8 mg. N)	6	12.0	0.68	2.6	
	7	9.5	−0.46	0.0	−46
l-Alanine (2.0 gm. containing 314.6 mg. N)	6	9.2	0.05	0.2	−19
	8	17.5	0.44	1.65	76
Unnatural forms					
d-Leucine (2.0 gm.)	6	15.9	0.66	1.46	16
	7	10.6	0.22	0.83	−6
d-Alanine (2.0 gm.)	6	15.2	27.8	104.2	−77
	8	7.9	19.0	71.3	16

The data in Table IV deal with the extra elimination of amino acid nitrogen and urea nitrogen, as well as the ammonia nitrogen elimination.

It has been observed frequently that after the injection of the unnatural isomer of an amino acid there is a considerable excretion of that form in the urine. The results with the unnatural form of alanine bear this out. As much as one-third of the injected nitrogen was eliminated. This was not the case with the natural isomer of alanine, and both forms of leucine were retained after injection, with only negligible loss in the urine.

The excretion of urea showed no significant changes. Such are not to be expected in short experiments, nor could conclusions be safely drawn about the excretion of urea when there were periods of marked diuresis due to the fluid administered.

Several experiments were carried out in which the HCl and the amino acid were in solution together and injected intravenously. The results in these experiments were similar in the amount of ammonia eliminated and the time relations in excretion to those reported with HCl administered by stomach tube.

DISCUSSION

The administration of hydrochloric acid by stomach tube to dogs is a stimulus to the formation and elimination of ammonia excreted in the urine as a part of the acid-neutralizing equipment of the body.

In control experiments the administration of acid alone resulted in regular increases in the ammonia elimination in those periods in which the urine volume gave evidence of the absorption of the acid administered. These conclusions are further strengthened by the experiments in which the same strength and amounts of hydrochloric acid were injected intravenously.

If the amino acids coming to the kidney in arterial blood serve as the precursors for the formation of ammonia for acid neutralization, the simultaneous administration of mineral acid to increase the stimulus to ammonia formation and the injection of additional amino acids should cause more ammonia formation and excretion than the injection of either one of them alone. This has been found to be the case. The injection of amino acids without any mineral acid to increase the acid load showed only a slight increase in ammonia output, and the administration of acid alone was accompanied by the excretion of an amount of ammonia that was considerably less than was found when both were given at the same time.

SUMMARY

1. Experiments with dogs indicate that there is an increased elimination of urinary ammonia following the intravenous injection of alanine and leucine.

2. Both optical enantiomorphs serve as precursors.

3. There is more urinary ammonia elimination following the injection of the unnatural (d) forms of these amino acids than with the natural forms.

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STUDIES ON THE PRODUCTION OF TAUROCHOLIC ACID IN THE DOG

V. METHIONINE SULFOXIDE*

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Since administration of methionine to bile fistula dogs had been followed by an increased output of taurocholic acid (1), it became of interest when methionine sulfoxide was prepared (2) to see whether or not this oxidized derivative might give rise to extra taurine. If the sulfoxide could be changed to taurine, then one might assume that oxidation could be an early step in the formation of taurine from methionine.

EXPERIMENTAL

Cholic acid is usually considered to be the limiting factor in the formation of taurocholic acid (3). However, when an excess of cholic acid over taurine is present in the liver of dogs, taurine becomes the limiting factor. In the experiments herein described we depleted the livers of female bile fistula dogs of their taurine by fasting and by feeding 6.7 milliequivalents of cholic acid daily. The cholic acid was given so that it might unite with any taurine in the liver and be excreted as taurocholic acid in the bile. On the 3rd day of this régime, 6.7 milliequivalents of methionine sulfoxide were dissolved in water and given with the cholic acid. Some administrations were made subcutaneously and some intravenously. If methionine sulfoxide could be changed to taurine, then an increased output of taurocholic acid should be observed after this procedure. The bile was collected at 24 hour intervals,

* A preliminary report of these studies was presented before the Thirty-fourth meeting of the American Society of Biological Chemists at New Orleans, March 13, 1940.

TABLE I

Urinary Sulfur Partitions and Taurocholic Acid Production Following Administration of Methionine Sulfoxide to Fasting Bile Fistula Dogs

Each animal was fed 2.8 gm. (0.213 gm. of S) of methionine sulfoxide = 6.7 milliequivalents of cholic acid daily.

Dog No.	Day	Weight	Total N	Total S	Sulfate S	Organic S	—SS— S	Taurocholic acid
		kg.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
54	1	14.1	11.77	674	431	243	10	3325
	2		8.67	501	344	157	10	2520
	3		6.90	460	298	162	9	2677
	4		6.31	500	325	175	9	2656*
	5	11.4	6.74	431	299	132	8	2357
57	1	17.7	7.25	452	338	114	7	2791
	2		5.72	382	271	111	5	2666
	3		6.43	490	324	166	3	2852†
	4	17.0	5.98	366	255	111	3	2719
59	1	14.5	4.67	240	135	105	6	2512
	2		3.91	222	120	102	9	1316
	3		4.18	346	167	179	6	2146†
	4	13.9	3.76	242	139	103	6	1274
60	1	10.5	6.34	369	244	125	8	3429
	2		5.76	311	202	109	6	2154
	3		5.62	446	253	193	6	2507*
	4	9.1	4.09	234	145	89	4	1244
61	1	12.3	7.31	219	118	101	6	892
	2		3.98	209	115	94	3	510
	3		2.52	272	109	163	2	570†
	4	10.9	2.80	197	106	91	3	324
61	1	12.3	6.66	326	184	142	4	955
	2		4.36	252	144	108	4	653
	3		3.86	379	182	197	2	1178‡
	4	11.0	3.40	238	131	107	3	747
62	1	11.4	5.95	394	277	117	7	3328
	2		4.93	279	179	100	6	1617
	3		5.16	449	280	169	5	2103*
	4	10.5	4.64	286	188	98	3	1371

6.7 milliequivalents of methionine sulfoxide (1.1 gm.) were dissolved in water and injected (*) subcutaneously, (†) intravenously, and (‡) intravenously 2 hours after cholic acid was fed.

just before the cholic acid was given. The urines were collected by catheterization.

The methionine sulfoxide was prepared according to Toennies and Kolb (2).[1]

C$_5$H$_{11}$NO$_3$S.   Calculated, S 19.33; found, S 19.10

The cholic acid was purchased from Riedel-de Haen.   The analytical methods used have been described previously (1).

Results

The first subcutaneous administration of methionine sulfoxide (Dog 54, Table I) was rather unsuccessful, for only 40 mg. of sulfur were recovered after 213 mg. had been given.   Little effect was seen on the sulfur content of either urine or bile.   The next administration (Dog 57) was made intravenously.   More than half the sulfur was recovered, most of which appeared in the urine.   About half the urinary sulfur had been oxidized to sulfate.   A slight rise in taurocholic acid production was observed, but it was not large enough to be significant.   Somewhat similar results were obtained when methionine sulfoxide was injected intravenously into Dog 61 the first time.   A slightly higher output of taurocholic acid was observed which was not large enough to permit drawing any conclusions.   All of the recovered urinary sulfur appeared in the organic fraction.

Four more experiments were performed with methionine sulfoxide, all of which produced consistent and definite results indicating three things: first, that administration of methionine sulfoxide may be followed by an increased output of taurocholic acid; second, that the dog has difficulty in oxidizing completely the sulfur of methionine sulfoxide, as shown by the relatively large proportion of urinary sulfur in the organic fraction; and third, that no disulfide sulfur appears in the urine as a result of metabolism of the sulfoxide.   Administration was made intravenously to Dogs 59 and 61 (second experiment), and subcutaneously to Dogs 60 and 62.   The second time methionine sulfoxide was given to Dog 61 it was not injected simultaneously with the feeding of cholic acid, but was given 2 hours later.   This was done to be

sure the cholic acid and the sulfoxide might be in the body at the same time, so that conjugation would be enhanced if such a reaction were possible.   No delay in subcutaneous administration was considered necessary, for absorption of cholic acid from the alimentary tract should proceed simultaneously with absorption of the subcutaneously administered material.

DISCUSSION

The results obtained on comparison of the oxidation of methionine (1) and of methionine sulfoxide by the dog are analogous to those observed on comparison of the oxidation of cystine with that of its partially oxidized derivatives (4); namely, that the more highly the sulfur is oxidized in the compound administered the more difficult becomes the oxidation of that substance to sulfate. The oxidized derivatives of both cystine (4) and methionine give rise to extra excretion of taurocholic acid, which requires oxidation of the sulfur to the sulfonic acid.   Since methionine and its sulfoxide may cause production of taurocholic acid, the sulfoxide may be an intermediate in the change of methionine to taurine.   This oxidation would necessitate a different path than that proposed (5) for the oxidation of methionine to sulfate, in which demethylation of methionine was a preliminary step in its metabolism.

No proof is yet available that methionine is actually changed to taurine.   It may be that methionine stimulates the formation of taurine in some manner, as by replacement of cystine, which may be changed to taurine.

SUMMARY

Bile fistula dogs were depleted of their taurine by fasting and by giving cholic acid daily.   When methionine sulfoxide was administered with the cholic acid, an increased output of taurocholic acid was observed.

A large portion of the extra urinary sulfur from the methionine sulfoxide appeared in the organic fraction, which indicates that the oxidation of methionine sulfoxide to sulfate is somewhat more difficult than the oxidation of methionine to sulfate.

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THE URINARY PYRUVATE IN THIAMINE DEFICIENCY*

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An increase in the so called bisulfite-binding substances in the blood of beriberi patients and experimental animals deficient in thiamine has been noted by many workers. One might expect a similar increase in the urine. This has been reported in thiamine-deficient rats by Banerji and Harris (1) and confirmed by Shils, Day, and McCollum who have further studied the factors influencing it (2).

Admittedly, a determination of the total bisulfite-binding substances lacks in specificity what it gains in simplicity. Since pyruvic acid is presumed to be the intermediate chiefly responsible for the increase, it would seem to be an improvement to determine this substance directly. This is especially true of determinations made on urine, since many normal urinary constituents will bind bisulfite. An important example is allantoin which occurs in rat urine in significant quantities and varies with a multiplicity of physiological changes, particularly those attendant upon the metabolism of thiamine-deficient animals.

This paper reports the results of studies of the pyruvate excretion in thiamine deficiency and some of the factors which influence it.

EXPERIMENTAL

Rats from our stock colony, 120 to 160 gm. in weight, were used for these experiments. The animals were fed a thiamine-free diet having the following per cent composition: casein (alcohol-

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extracted) 18.0, corn-starch 56.0, butter (washed) 5.0, autoclaved yeast (Harris) 15.0, cod liver oil 2.0, salt mixture (Osborne-Mendel (3)) 4.0. In addition, after the 6th week or when food consumption diminished, each animal was fed twice weekly 0.1 cc. of a mixture containing 30 γ of pyridoxine, 30 γ of riboflavin, and 10 γ of thiamine.[1]

For the investigation of the urinary pyruvate, groups of ten animals were studied at weekly intervals. These were placed in individual metabolism cages with facilities for collection of the urine. A measured quantity of the depletion diet was allowed with water *ad libitum*. At the end of 24 hours the food was removed and the consumption recorded. Urine collections were made by dilution to 20 cc., representing a 24 hour sample. The animals were then fasted for 24 hours and the urine collections made as before.

The urinary pyruvate was determined by a modification of the specific hydrazine procedure of Lu (4). To a 15 cc. cone point centrifuge tube there were added 1.5 cc. of 10 per cent trichloroacetic acid. A 0.5 cc. sample of urine was added and the contents mixed by rotation. 1 cc. of a 0.1 per cent solution of 2,4-dinitrophenylhydrazine in 2 N HCl was then added and mixed. Extraction of the hydrazones and unchanged hydrazine with ethyl acetate was carried out as in the original method, the ethyl acetate extracts being transferred to a second centrifuge tube. Rapid separation of the ethyl acetate layer was accomplished by centrifugation. The pyruvic hydrazone was extracted as in the original method with three successive 2 cc. portions of 10 per cent sodium carbonate. The combined carbonate extracts (6 cc.) were then poured into a graduated colorimeter tube and 2 N sodium hydroxide was added to bring the volume to 10 cc. The contents were then mixed by inversion and readings made after 10 minutes in a Klett-Summerson photoelectric colorimeter (5) with a No. 54 filter. The colorimeter was previously calibrated for pyruvic acid by the use of a lithium pyruvate standard prepared according to Wendel (6).

As an index of adequate excretion as well as of the validity of the urine dilutions, simultaneous determinations of 24 hour

[1] These supplements were crystalline products obtained from Merck and Company, Inc.

creatinine excretions were made. Pyruvate values were considered valid only when accompanied by a normal urinary output as determined by this method. In the determination of creatinine, a modification of the micromethod of Shaffer (7) was employed; a Klett-Summerson photoelectric colorimeter with the No. 54 filter was used.

Results

The average pyruvate excretion of the male and female rats subsisting on the thiamine-deficient diet is recorded in Table I. From these data it will be noted that a definite increase in pyruvate has occurred: for the male animals, from a mean of 1.84 ± 0.05 mg. per day at the 6th day of depletion to 3.57 ± 0.34 mg. per day at the 43rd day; for females, an increase from 1.50 ± 0.11 mg. per day at the 3rd day to 2.90 ± 0.21 mg. per day at the 45th day. The values for the quotient of the mean differences to the probable error of the mean differences exceed 3.00, indicating statistical validity.

In order to evoke this excretion it is necessary that the animal be well nourished. As the lowered values for the fasting period indicate, the state of alimentation of the animal markedly affects the pyruvate output. This fact was also reported for the urinary bisulfite-binding substances by Shils, Day, and McCollum (2). Presumably the level of liver and muscle glycogen is an important factor. The relation to food intake is not, however, strictly quantitative, for although a markedly diminished intake (less than 5 gm. in 24 hours) will lower the expected output, a normal intake (10 gm. or more) does not produce a proportionate increase. For the fasting period the increased excretion is in the same direction as that observed for the collection period during which food was allowed.

According to the needs of the animal thiamine feeding even in small dosages produced a temporary lowering of the output of pyruvate. This was observed for the males between the 29th and 36th days and similarly in the females between the 32nd and 39th days. Suspension of the supplement was reflected by a return to elevated levels.

As recommended by Banerji and Harris (1) for evaluation of the urinary bisulfite-binding substances as a reflection of the

thiamine status of the animal, elevated values should respond to dosage with thiamine.   As the results summarized in Table II indicate, this has been observed with urinary pyruvate.

TABLE I

Excretion of Pyruvate by Rats during Prolonged Thiamine Deficiency

Days on diet	Average body weight	Average food intake on test day	Urinary pyruvic acid*	
			Food *ad libitum* for 24 hrs.	During first 24 hr. fast
Male rats				
	gm.	gm.	mg.	mg.
6–7	141.7	15.2	1.84 ± 0.05	0.70 ± 0.05
15–16	136.7	14.8	2.26 ± 0.08	0.95 ± 0.09
22–23	135.6	12.5	2.68 ± 0.14	1.13 ± 0.08
29–30	133.9	13.2	3.20 ± 0.27	1.45 ± 0.07
36–37†	129.7	11.0	2.76 ± 0.26	1.02 ± 0.06
43–44‡	133.2	7.7	3.57 ± 0.34	1.24 ± 0.08
M.D.:S.E.M.D.§ (6–7 and 43–44 days)			5.00	5.75
Female rats				
3–4	132.8	12.9	1.50 ± 0.11	0.54 ± 0.04
10–11	130.3	16.2	1.92 ± 0.10	0.84 ± 0.04
17–18	128.5	15.2	3.90 ± 0.42	1.08 ± 0.06
24–25	124.8	13.5	2.44 ± 0.16	0.93 ± 0.08
31–32	129.5	9.7	2.52 ± 0.18	1.16 ± 0.04
38–39†	130.2	7.7	2.22 ± 0.15	0.74 ± 0.05
45–46‡	125.2	7.5	2.90 ± 0.21	0.97 ± 0.06
M.D.:S.E.M.D. (3–4 and 45–46 days)			5.94	5.95

Each value is the average of ten experimental animals.

* Including the standard error of the mean calculated as follows: $\sqrt{(\Sigma\ d^2/n)}/\sqrt{n}$.

† Thiamine feeding during the previous 7 day period.

‡ No thiamine feeding during the previous 7 day period.

§ Ratio of the mean difference to the standard error of the mean difference.   When this value exceeds 3.00, the results are considered significant.

In these experiments a group of male and female animals was depleted for long periods during which elevated pyruvate levels were maintained.   Small dosages of thiamine (20 to 30 γ per week) did not produce extensive diminution in these levels.   From the 78th day of depletion to the 98th day, 600 γ per week were fed to

each animal. This produced a significant drop in the pyruvate output: in the males, from 5.34 ± 0.85 on the 70th day to 2.57 ± 0.26 on the 98th day, and in the females, from 3.46 ± 0.25 to 2.55 ± 0.14 for the corresponding period.

A consistent sex difference in the pyruvate excretion appears to prevail in the twenty series of the experiments summarized in Tables I and II in all cases except two (females, 17 to 18 days, Table I), which we consider an aberrant result. This variation appears both during alimentation and following fasting.

TABLE II

Response of Pyruvate Excretion to Thiamine Feeding

Days on diet	Sex	Average weight	Thiamine administered during wk. previous to collection	Average food intake on test day	Urinary pyruvic acid*	
					Food *ad libitum* for 24 hr. period	During first 24 hr. fast
		gm.	γ	*gm.*	*mg.*	*mg.*
63–64	F.	123.0	30	15.6	4.00 ± 0.21	1.07 ± 0.16
	M.	125.6		16.8	5.15 ± 0.79	1.25 ± 0.24
70–71	F.	124.2	30	11.6	3.46 ± 0.25	1.20 ± 0.14
	M.	128.4		12.0	5.34 ± 0.85	1.50 ± 0.02
77–78	F.	133.2	30	5.4	2.83 ± 0.12	1.00 ± 0.01
	M.	132.2		8.6	3.95 ± 0.61	1.15 ± 0.01
98–99	F.	140.7	600	15.0	2.55 ± 0.14	0.88 ± 0.10
	M.	138.4		14.0	2.57 ± 0.26	1.00 ± 0.11
	F.	M.D.:S.E.M.D. (70–99 days)			3.18	1.86
	M.				3.13	4.47

Each value is the average of five experimental animals.

* Including the standard error of the mean.

DISCUSSION

These results indicate that in animals on a thiamine-deficient diet some estimate of the nutritional status with respect to thiamine is reflected by the level of urinary pyruvate. The relation to the state of alimentation is not surprising and it is of interest to note that the sex differences observed coincide well with those reported for liver glycogen by Deuel *et al.* (8–10).

Thiamine supplements adequate for minimal growth are not sufficient to abolish completely the heightened pyruvate output. In fact, one obtains more constantly elevated values when animals

are maintained in this subacute avitaminotic state rather than in an acute, and often moribund, condition.

As depletion proceeds, elevated levels of pyruvate are noted even during the 24 hour fasting period. This might be due to the fact that a previously higher level requires a longer period for excretion or that there is a retention of the precursors of pyruvate, causing a retarded and hence prolonged formation of this metabolite. The former suggestion is not enhanced by the results of our own unpublished observations on the excretion of pyruvate, both in normal and deficient rats. These results indicate a rather rapid excretion of excess pyruvic acid. Thus, when sodium pyruvate was administered by stomach tube in a dose of 118 mg. per 100 sq. cm. of body surface, as much as 50 per cent of that administered was excreted during the first 24 hours. This dosage proved toxic in several cases. Not until the dose was lowered to a level of 5 mg. per 100 sq. cm. did no augmentation of the expected pyruvate output occur.

SUMMARY

1. The excretion of pyruvate in the urine of rats increases as the depletion of the thiamine reserve proceeds. It is reduced by optimal thiamine administration.

2. The level of excretion of pyruvate is related to the state of alimentation of the animal during the period of the urine collection.

3. Urinary pyruvate appears to be higher in males than in females studied under identical conditions with respect to depletion of thiamine.

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THE INFLUENCE OF SERUM PROTEINS ON THE SPEC-
TROPHOTOMETRIC ABSORPTION CURVE OF PHENOL
RED IN A PHOSPHATE BUFFER MIXTURE

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The adsorption of phenol red by serum proteins is of great in-
terest to investigators studying the mechanism of secretion by the
mammalian kidney and also to those employing this dye for the
estimation of blood pH. This phenomenon was demonstrated
first by de Haan (1) and then by Marshall and Vickers (2) who
measured the amount of dye which was filtrable through collodion
membranes impermeable to protein. Later the binding of dye by
protein was studied in this manner by Grollman (3), Shannon (4),
Goldring, Clarke, and Smith (5), and Smith and Smith (6). In
most of these studies ultrafiltration was carried out at 37° under
a CO_2 tension of 40 mm.

In the determination of serum pH Cullen (7) diluted the serum
21-fold with saline containing phenol red. He assumed that the
protein effect on the dye was negligible at this dilution or was
reduced to a constant factor which is included in the C correction
used in the method. (C is the factor subtracted from colorimetric
readings on the diluted sample at 20° to give the pH of the un-
diluted serum at 38°.) Robinson, Price, and Cullen (8) compared
the values obtained for dog serum by the colorimetric pH method
with those obtained by the electrometric pH method and showed
that the C correction is influenced by the amount of protein in the
serum. It was observed at the time of this study that some sera
produced marked changes in the color tones, and that the concen-
tration of the dye appeared to be greatly reduced. Rabbit serum
always gives a color tone which is distinctly more purple than the
purplish red color of the phosphate buffer standards. Marshall

and Vickers (2) record a marked species difference in the amount of dye adsorbed, the blood serum of rabbits binding much more than that of dogs. It occurred to us that this adsorption of dye by the serum proteins might be utilized as a means of characterizing these proteins, and might also aid in the detection of changes in the protein during pathological conditions.

This report presents a study of the influence of human, rabbit, or dog serum on the absorption curve of phenol red in phosphate buffer solutions. The variation in these curves shows the existence of a distinct species difference and will help to account partially at least for the fact that the C corrections of the Cullen method are not similar for all species. Total serum protein and serum albumin determinations were made on most of the samples of sera used in the study. These values were correlated with the density values of the color solutions in order to determine the existence of any relationship between the two.

Grollman (3) observed that at the pH of normal blood practically all of the adsorption was due to the albumin fraction. Our results to be presented confirm this finding. Grollman also emphasized the importance of pH control. Therefore, in a study of the protein effect on phenol red, it would be ideal if the serum could be added to a color solution in quantities sufficient to show a measurable color change without altering the pH of the color solution. Also this solution should have approximately the ionic strength of the serum in order that no appreciable change in the activity coefficient of the dye will occur. These conditions have been approached in the mixtures which we have studied.

<div align="center">EXPERIMENTAL</div>

When 1 cc. of blood serum was added to 20 cc. of M/15 phosphate buffer-dye mixture (Na_2HPO_4-KH_2PO_4), the pH as determined by the electrometric method was in most cases within 0.01 unit of that of the dye-phosphate mixture to which 1 cc. of water had been added. However, visual observation and a comparison of the absorption curves of the two solutions indicated that the equilibrium of the dye had been disturbed. Since the ionic strength and pH of the solution remained practically constant, this alteration is due to a constituent of the serum which is shown to be protein. In this communication spectrophotometric absorption

curves obtained by the introduction of blood serum into phenol red-phosphate buffer solutions are compared with the curves resulting when no serum is introduced. All of the work was carried out in a temperature-controlled room at $25° \pm 1°$.

Phosphate Buffer Solutions—$M/7.5$ disodium hydrogen phosphate solution, 18.94 gm. of anhydrous salt, Na_2HPO_4, per liter, and $M/7.5$ potassium dihydrogen phosphate solution, 18.16 gm. of salt, KH_2PO_4, per liter, were used. Merck's Blue Label Sörensen salts were used without further purification. The solutions were stored in a refrigerator without preservative and were discarded for new ones as soon as any mold growth appeared. From these stock solutions a $M/7.5$ buffer mixture was made by measuring the proper amounts of each and mixing thoroughly.

Preparation of Phenol Red Solutions—A 0.04 per cent aqueous solution of phenol red (Hynson, Westcott, and Dunning) was was prepared according to the directions given by Clark (9). It was stored in a refrigerator when not in use. From this stock a 0.0016 per cent solution was made up weekly by diluting 8 cc. to exactly 200 cc. with CO_2-free distilled water.

It was important that the phenol red used in this study should be of the highest purity. In $M/15$ phosphate buffer solutions the dye gave a maximum absorption at 560 mμ when the scale of our spectrophotometer had been carefully set at 589 mμ with the sodium lines. The absorption curve of our dye was identical with that for a sample of a specially prepared dye which had been kindly sent to us by Dr. Julius Sendroy, Jr. The latter sample was from the stock which he and Dr. Hastings had used in their studies on the apparent activity coefficients of acid-base indicators (10).

It was desirable to determine the pK' value of our dye as it would also give an index of its purity and would help to evaluate our optical and pH measurements in relation to the work of other investigators. Optical density values at 560 mμ (D_{560}) of a number of phenol red-phosphate buffer mixtures varying in pH from 7.27 to 8.07 were compared with the density of a phenol red-sodium hydroxide solution in which all of the dye is present in the alkaline form ($D_{m\ 560}$). The final concentration of dye in all of the solutions was 0.001 per cent. At pH ranges from 7.45 to 8.20 there exists a linear relationship between the density and pH values. When

the same concentration of dye is placed in 0.001 N HCl the density value at 560 mμ measured under the same conditions was 0.005, which is within the experimental error of our readings. Therefore the amount of the density value at this wave-length due to the acid form of the indicator is so small that it can be neglected in our calculations. The pK' values were calculated by the formula

$$pK' = pH - \log \frac{\alpha}{1 - \alpha}$$

where α is the ratio of the observed density value D_{560} to the maximum density value $D_{m\,560}$. The pK' values of the dye at 25° for each solution are listed in Table I. The average pK' value of this series is 7.736. Using the same standardization, Hastings and Sendroy (11) by colorimetric readings found that the pK' value of phenol red at 38° was 7.65 and at 20° was 7.78. These values would indicate a pK' at 25° of 7.74. They later revised their figure at 20° (10) to 7.80 which would mean a value 0.02 pH higher. After consideration of the number of variables in the determination, it is apparent that our work agrees satisfactorily with that of these workers.

Preparation of Test Color Solutions for Spectrophotometric Readings and pH Measurements—Since most of the experiments were conducted at a constant pH, it was desirable to use a phosphate buffer mixture at a reaction which would produce a color value close to that observed when blood serum of normal reaction is diluted 1:20 in saline. In the case of dog serum this color value is given by M/15 phosphate buffer solutions at pH 7.70 to 7.75, but for human sera the value is given by solutions about 0.10 pH lower. As the pH of a M/15 phosphate buffer mixture containing 9 parts of Na_2HPO_4 and 1 part of KH_2PO_4 at 25° has a pH value at the pK' value of the dye, a M/7.5 buffer mixture with this ratio of alkaline and acid salt was used as a stock solution. 10 cc. of this stock were mixed with 10 cc. of 0.0016 per cent phenol red solution in a test-tube of convenient size (22 × 150 mm.). 1 cc. of blood serum was added to this solution and, after being mixed well with a stirring rod, the contents were allowed to stand for 1 hour. A control tube without dye was prepared at the same time by mixing 10 cc. of M/7.5 buffer solution, 10 cc. of distilled water, and 1 cc. of serum.

Portions of these solutions were placed in identical optical cells of 15 mm. depth, the serum-dye-phosphate solution being placed in one cell and the serum control in the other cell. These cells were made especially for us by the Bausch and Lomb Optical Company with the feature that disks with optically plane surfaces were held against the glass tubing by screw-caps. This construction enabled us to replace the disks if they became scratched during handling or fogged by the potassium phosphate solutions. The optical density values were obtained with the Bausch and Lomb

TABLE I

pK′ (25°) of Phenol Red in Phosphate Buffer Solutions

Final concentration of dye = 0.001 per cent (0.0282 m$\times$ per liter); final concentration of PO_4 = 0.0667 $\times$; density values were observed at wavelength 560 mμ; depth of solution = 15 mm.

Solution	pH	Density (D_{160})	$\alpha = \frac{D_{160}^*}{D_{m\,160}}$	$\frac{\alpha}{1-\alpha}$	$\mathrm{Log}\frac{\alpha}{1-\alpha}$	pK′
A	8.066	1.79	0.682	2.145	0.331	7.735
B	7.819	1.44	0.549	1.217	0.085	7.734
C	7.756	1.345	0.512	1.049	0.021	7.735
D	7.677	1.23	0.469	0.883	$\bar{1}$.946	7.731
E	7.565	1.057	0.403	0.675	$\bar{1}$.829	7.736
F	7.460	0.91	0.347	0.531	$\bar{1}$.725	7.735
G	7.367	0.78	0.297	0.422	$\bar{1}$.625	7.742
H	7.269	0.665	0.253	0.339	$\bar{1}$.530	7.739
Average...						7.736

* $D_{m\,160}$ = density of phenol red solution (0.001 per cent) in 0.005 $\times$ NaOH = 2.625. $D_{a\,160}$ = density of phenol red solution (0.001 per cent) in 0.001 $\times$ HCl = 0.005 (approximate).

universal spectrophotometer, and in every case the density at a particular wave-length was an average of the reading made with the color solution in one beam of light and of a second reading with the color solution in the other beam. The collimator slit of the spectrometer was set at 1.0 on the arbitrary scale of the instrument and the ocular slit was set at a width corresponding to about 3 mμ. For the curves (515 to 610 mμ) readings were generally made at intervals of 5 to 10 mμ, except near the peaks of absorption where they were also made at intermediate points.

The pH of the color solution was determined immediately in a

glass electrode vessel of the MacInnes-Belcher type (12) with the use of silver-silver chloride and saturated calomel electrodes to complete the circuit. A liquid junction was formed with saturated potassium chloride, no correction being made for the liquid junction potential. A Leeds and Northrup ion potentiometer was used in the electrical circuit with amplification of the potentiometer unbalance by means of a General Electric Pliotron FP-54 tube. The lamp, scale, and focusing lens of a Leeds and Northrup galvanometer (No. 2420C) were removed, and a lamp and scale reading device supplied by the same manufacturers was introduced which focused the movement of the galvanometer at a distance of 1 meter. This apparatus had a sensitivity sufficient to detect an unbalance of 0.1 millivolt.

Standardization of the glass electrode was effected each day with the use of M/15 phosphate buffer mixtures, 0.05 M potassium acid phthalate solution, or 0.1 N acetic acid buffer mixtures. The phosphate and acetic acid mixtures had been standardized first with a hydrogen electrode system in which the pH of 0.1 N HCl was assumed to be 1.080 at 25°. On this basis the pH value of 0.05 M potassium acid phthalate was 4.02, which is about 0.02 unit higher than the value obtained by MacInnes, Belcher, and Shedlovsky (13). However, when we assumed their value for the acid phthalate buffer, we obtained values for the acetic acid mixtures which duplicated theirs. Since we were not primarily interested in absolute values, but only in the constancy of our values, no adjustment has been made of the pH for these discrepancies.

The total serum protein and serum albumin determinations were made by the methods previously described by us (14). In order to study the effects of higher concentrations of protein, ultrafiltration of serum through cellophane membranes under a pressure of approximately 150 pounds per sq. inch was carried out in the apparatus previously described (8). Solutions were also made of diluted serum in which saline was used as a diluent.

Simultaneously with the preparation of the serum-dye-phosphate solutions, a mixture was prepared in which the 1 cc. of serum was replaced by 1 cc. of water. Density and pH determinations were made on this mixture exactly as described above in order that the extent of the change effected by serum on the density value and on the pH might be ascertained.

Typical Absorption Curves—The curves for mixtures containing dog serum are shown in Fig. 1 (Curves B and D). Curves A and C are those of the phenol red-phosphate buffer mixtures without serum at pH 7.74 and 7.54 respectively, and illustrate the change in density which occurs with change of reaction. For
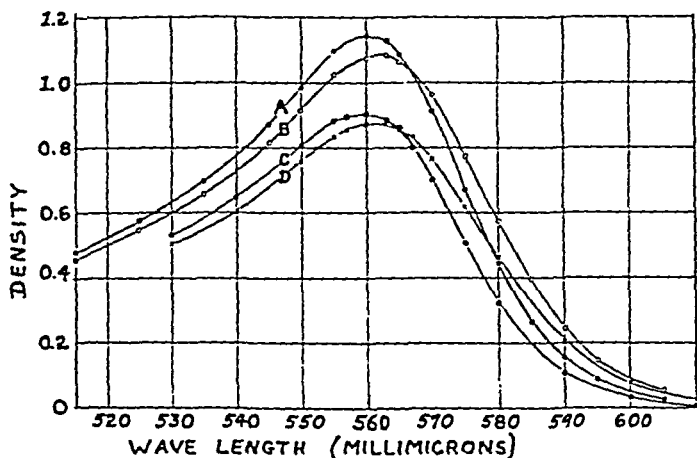

FIG. 1. Absorption curves of phenol red in phosphate buffer mixtures with and without the addition of dog serum. The density readings of the mixtures were made with the Bausch and Lomb spectrophotometer in cells at a depth of 15 mm. The final concentration of phenol red in each mixture was 0.00076 per cent. Solution A, 10 cc. of 0.0016 per cent phenol red solution + 10 cc. of M/7.5 phosphate buffer (Na_2HPO_4-KH_2PO_4) mixture + 1 cc. of water; pH of solution = 7.740. Solution B, the same as Solution A except that 1 cc. of dog serum was added instead of water; pH of solution = 7.732. Solution C, the same as Solution A except that a phosphate buffer mixture of a lower pH value was used; pH of solution = 7.540. Solution D, the same as Solution B except that the phosphate buffer mixture of Solution C was used; pH of solution = 7.534.

example, the density of the more alkaline solution at the point of maximum absorption, 560 mμ, is 1.14, while the density of the other solution at the same wave-length, 0.2 pH lower, is 0.90. Since the density is a linear function of the pH in this region, a change of density of 0.01 signifies a pH change of 0.0083.

Curves B and D illustrate the effect of adding 1 cc. of dog serum

to the dye-phosphate mixtures used for Curves A and C respectively. No appreciable change in the color of these solutions could be observed with the eye alone, but the peaks of the absorption curves were lowered and shifted toward the red end of the spectrum after the introduction of serum, even though the pH was altered only 0.008 and 0.006 respectively towards the acid side. The extent of the shift was greater in the more alkaline buffer mixture. Furthermore, the curves of the serum-dye-phosphate buffer mixtures showed increased absorption above 565 mμ and decreased absorption below this wave-length in contrast to the corresponding mixtures without serum. An examination of many samples of dog sera in this manner showed that the two curves (one with and one without dog serum) crossed each other at about 565 mμ. If the pH is calculated from the spectrophotometric readings at this wave-length, a good agreement is obtained with the pH value determined by the electrometric procedure and also with the readings obtained by direct visual comparison with dye-phosphate buffer standards employing the Walpole comparator principle. These results would indicate that the C correction for dog serum in the Cullen colorimetric pH method was due mostly to dilution and to change in temperature.

The absorption curves of the dye-phosphate buffer mixture at pH 7.74 following the addition of human or rabbit serum were distinctly different from the curves obtained with dog serum. In Fig. 2 are shown the positions of curves after the addition of normal human or rabbit serum to the dye-phosphate mixtures in relation to that of the dye-phosphate mixture alone as indicated by the dotted line. While the pH of the three solutions was within 0.01, there was a great reduction of the absorption values around the region of 560 mμ after the addition of these sera. This effect, readily observed with the eye, gave the impression that either the reaction had been markedly lowered or that the dye concentration had been reduced. The density value at 560 mμ for the mixture containing human serum was much lower than that observed for dog serum but was well above that for rabbit serum. Therefore when Cullen found that the C correction for human plasma or serum was less than that for dog, no doubt most of this difference was due to the greater adsorption effect of human serum. Unlike the curve for dog serum, there was no shift noticeable with human

serum. When samples of human serum were concentrated by ultrafiltration from a normal value of about 7 gm. per 100 cc. to a high value of 12 to 15 gm. per 100 cc., a greater absorption was observed in the regions above 560 mμ together with a marked flattening of the peaks of the curves. The addition of the ultrafiltrate of serum caused no change in the density or pH values. The shift effected by rabbit serum was more marked than that found for dog serum.
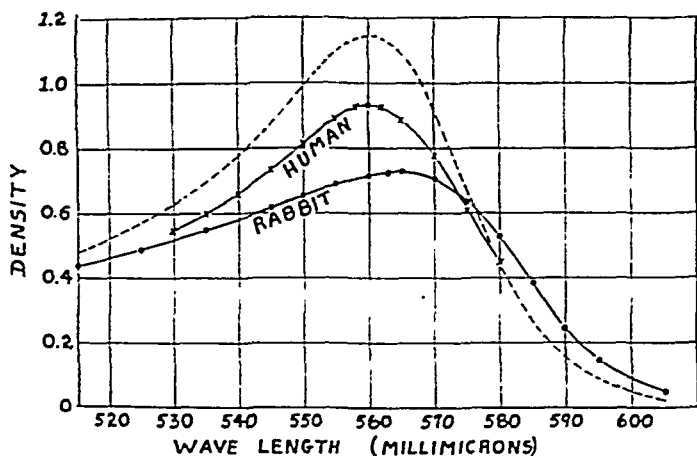

Fig. 2. Effect of human and rabbit serum on the absorption curve of phenol red in phosphate buffer mixture. The solutions contain the same phosphate buffer and phenol red concentrations as those used in Fig. 1. 1 cc. of human or rabbit serum was added to 20 cc. of buffer-dye mixture. The pH of the solution containing human serum was 7.741 and of that containing the rabbit serum was 7.733. The dotted line is the curve for the buffer-dye mixture without the blood serum but with 1 cc. of water added to make the dilutions the same. The pH of the latter solution was 7.74.

These curves are representative of a number of determinations which in all cases showed a distinct difference in dog, rabbit, and human sera. While the blood sera of other species were not studied intensively, it is felt that each species may show a characteristic effect. One determination on the serum from a pig showed a slight increase in the density value at 560 mμ, a shift of the position of the peak toward the red portion of the spectrum, and a

greater absorption in this part of the spectrum. One obtained the impression on visual observation that the dye concentration had been increased.

· *Observations with Alkaline Solutions*—The adsorption effects described above are observed only in reactions close to neutrality. When human, rabbit, or dog sera are diluted 1:20 in a phenol red-sodium hydroxide solution, no adsorption of the dye takes place; that is, the density values agree within experimental error with the values of the same solution without serum. A representative experiment is illustrated in Table II in which the serum of a normal rabbit was added to the mixture. Furthermore, the presence of phosphate in an alkaline solution does not modify the results. The substitution of a $M/15$ phosphate buffer mixture at

TABLE II

Effects Observed on Optical Density after Addition of Rabbit Serum to a Solution of Phenol Red in 0.005 N Sodium Hydroxide

Depth of solution = 10 mm.

Wave-length, $m\mu$.	530	540	550	555	558	560	562	565	570
Solution A	0.79	0.99	1.26	1.37	1.41	1.41	1.38	1.31	1.07
" B	0.80	0.99	1.25	1.38	1.41	1.41	1.39	1.32	1.08

Solution A = 10 cc. of 0.0016 per cent phenol red + 10 cc. of 0.01 N NaOH + 1 cc. of water. Solution B = 10 cc. of 0.0016 per cent phenol red + 10 cc. of 0.01 N NaOH + 1 cc. of serum.

a pH of 11.2 ($Na_3PO_4 : Na_2HPO_4 = 1$) for the sodium hydroxide solution caused no adsorption of dye with the addition of serum. No permanent alteration of the protein which would affect its action toward phenol red takes place in the alkaline solution. The following experiment confirms this fact. Rabbit serum was added to the dye-sodium hydroxide mixture in the same amounts as employed in the previous experiments. The density readings. made on this solution at the end of an hour showed no decrease in value when compared with the control sample of water-dye-sodium hydroxide. A phosphate buffer solution was then added to this mixture and also to the control tube which brought the final pH of these solutions down to approximately 8.2. At this reaction the density values of the tube containing the serum were

far below those of the control tube, and the absorption curve showed the typical shift of the position of maximum absorption as described above.

Influence of Buffer on Adsorption Effect—A number of experiments were carried out in which the phosphate buffer solutions were replaced by a solution of the veronal buffer system introduced by Michaelis (15). This latter was prepared by adding 0.1 N HCl to 0.1 M sodium diethylbarbiturate (sodium veronal) solution until, with the addition of the same concentration of
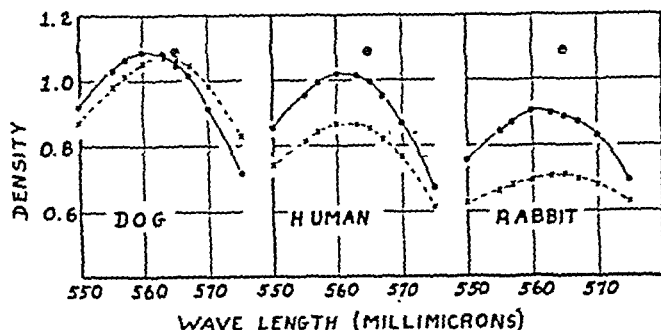

FIG. 3. Absorption curves of serum-dye-buffer mixtures, similar to those in Figs. 1 and 2, in which the effect of the veronal buffer is compared with that of the phosphate buffer. In each case 1 cc. of serum was added to 20 cc. of the buffer-dye mixture. The optical density values at 565 mμ of the phosphate-dye mixtures + 1 cc. of water were identical with those of the veronal-dye mixture + 1 cc. of water; the position on the graphs is indicated by the symbol ⊖. The pH values of the serum-dye-phosphate mixtures, the dotted curves, for dog, rabbit, and human serum respectively were 7.736, 7.737, and 7.736. The pH values for the corresponding serum-dye-veronal mixtures were 7.825, 7.827, and 7.826.

phenol red solution, the optical density at 565 mμ was exactly the same as that of the phosphate mixture at pH 7.74. Although the intensity of the colors of these two buffer mixtures was the same, the pH of the veronal solution was 7.83. The comparative absorption curves obtained when normal rabbit, human, or dog sera were added to these systems are presented in Fig. 3. The adsorption of phenol red by serum was never as great in the presence of the veronal buffer mixtures as it was in the presence of the phosphate buffer mixtures. Moreover, no appreciable shift of

the peak of absorption occurred with either dog or rabbit serum in the dye-veronal mixture. From these experiments we must conclude that the buffer system itself plays an important rôle in the extent of the adsorption of phenol red by blood serum.

Adsorption Effects of Serum Albumin and Globulin—From the studies of Grollman (3) and Smith and Smith (6) on undiluted serum it is well recognized that the adsorption of this dye was due to the albumin fraction. In the course of this study we prepared a number of samples of serum albumin and serum globulin by neutral salt fractionation. Solutions of globulin in saline with a concentration of about 5 gm. per 100 cc. were added to the dye-phosphate mixture in the same manner as the whole serum. The results indicated that the globulin of rabbit, dog, or human sera does not combine with the phenol red, for the absorption curves of the dye-buffer mixture were not altered by the addition of the globulin solutions. However, the albumin solutions with about the same concentration of protein gave the comparable effects observed when the whole serum of dog, rabbit, or human blood was employed. Our experiments therefore lead to the conclusion arrived at by the above workers.

The quantitative relationship between the amount of phenol red adsorbed and the concentration of the serum protein was studied. Since the point of maximum absorption of most of the rabbit serum-dye-phosphate mixtures occurs in the neighborhood of 565 mμ, we have plotted in Fig. 4 the relationship of the density values at this wave-length to the albumin concentration of the color solution (as mg. per cc. of color solution). In a series of samples from human sera there are some determinations on the sera of patients with the nephrotic syndrome, in which the serum protein level is greatly reduced due primarily to the loss of large quantities of protein, mostly albumin, in the urine. Investigators employing various procedures have shown that the protein in the blood stream of these patients has been modified from that of the normal individual. The density values of the mixtures made from the sera of these patients were identical with those obtained on normal sera diluted to the same protein concentration, which would indicate that, as far as ability to adsorb phenol red was concerned, the albumin was identical with that from the sera of normal human beings. Likewise determinations

on pathological sera of many other types never showed any deviation from the pattern given by normal human sera. The points at 0 concentration of protein (Fig. 4) are those obtained from the phosphate-dye mixtures to which 1 cc. of ultrafiltrate had been added. The higher concentrations of protein indicated are those obtained by ultrafiltration of the serum previously mentioned.

The decrease in the density value at wave-length 565 mμ is directly proportional to the rabbit albumin concentration from 0 to 1 mg. per cc. Above the latter concentration the rate of change
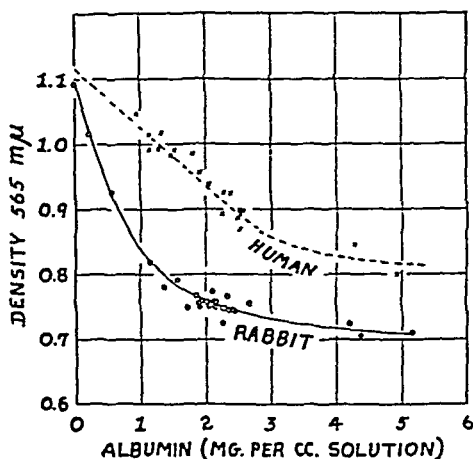
Fig. 4. The relation of the density values at 565 mμ to the albumin present in the phosphate buffer-dye mixture. The dilution of the serum is the same as that of previous experiments. The concentration of albumin in the original serum may be calculated, gm. of albumin per 100 cc. of serum = mg. per cc. of color solution $\times$ 2.1. The pH of all the mixtures was 7.74.

per gm. of protein becomes less, so that when a normal rabbit serum is concentrated to double its albumin concentration the decrease in the density value is almost within the error of the measurements. However, with the increase in protein concentration, the absorption curve shifts more toward the red end of the spectrum. The slope of the curve for human serum (Fig. 4) is much less than that of the curve for rabbit serum. A linear relationship between density and albumin values exists between 0 and 3 mg. per cc. of albumin. Above 3 mg. the change in

density value with albumin concentration decreases to a marked degree.

Grollman (3) found that the combination of phenol red and plasma proteins followed closely the adsorption isotherm $x/m = Kc^{1/n}$, where x equals the amount of dye adsorbed on m gm. of protein, c is the concentration of free dye, and k and n are constants. Goldring, Clarke, and Smith (5) show that their experiments with undiluted human plasma satisfied this relationship. We carried out a series of experiments by our procedure to determine whether or not this empirical law of Freundlich was obeyed. When rabbit serum was added to the M/15 phosphate buffer mixtures containing increasing amounts of phenol red, not only was the percentage of dye adsorbed decreased, but also the displacement of the position of maximum absorption was reduced. With increasing concentration of dye the points of maximum absorption more closely approached 560 mμ as found in the absence of serum. However, the shifting of the position of maximum absorption shown in Fig. 2 caused errors which made our calculations absurd. For example, in one of our experiments when we based our calculations on density values at 560 mμ, the percentage of bound dye was 17.6, and when we used the values at 565 mμ the percentage of bound dye was only 15.8. Therefore, the procedure used in our experiments cannot be used directly to determine the validity of this law. The optical system and the method were adequate because, with wide variations of dye concentration in phosphate buffer-phenol red mixtures, a constant value for the specific transmissive index was always obtained. From these experiments it is realized that with change of rabbit and dog albumin concentration, the change in density value is also accompanied by a shift in the absorption curve.

From these observations it may be concluded that the adsorption of phenol red by serum albumin will influence the magnitude of the C correction in the colorimetric determination of the pH of human and rabbit serum. As dog serum albumin, on the other hand, adsorbs practically none of the dye, the C correction in this case consists principally of the effects of dilution and of shifts in the values of the pK' of carbonic acid and protein with changes in temperature. When serum is diluted 1:20 in CO_2-free saline the change in reaction is toward one of greater alkalinity. The loss

of dye concentration by adsorption would lessen this dilution effect and give lower C corrections. The adsorption phenomenon no doubt explains the differences in C correction of the sera from various species which was observed by Cullen (7). The average value of the correction, 0.34, is greater for dog serum than for human serum, 0.23. The use of the colorimetric method for rabbit serum has always been unsatisfactory, as the tone of the serum-saline-dye mixture appears very different from that of the phosphate buffer color standard. However, the C corrections reported for rabbit serum by various investigators have always been lower than those for human serum, which might be expected from our absorption curves. Robinson, Price, and Cullen (8) observed that the magnitude of the C correction, both in human and dog sera, is related to the concentration of the protein in the serum. Our observations in this report would indicate that adsorption of dye by dog serum albumin is not a part of this effect.

SUMMARY

1. Blood serum may be added in the proportion of 1:20 to a $M/15$ phosphate buffer mixture with practically no change in the pH of the mixture. This system containing phenol red at a pH of 7.74 was utilized to study the adsorption effects of human, dog, and rabbit sera on the dye. Spectrophotometric absorption curves were determined for the region between 500 and 600 mμ.

2. The decrease in the optical density values in the region of 560 mμ was greatest with rabbit serum, much less with human serum, and very slight for dog serum. Rabbit and dog sera caused a shift of the position of maximum absorption toward the red end of the spectrum.

3. This effect is due almost entirely to the albumin concentration of the serum and in the case of rabbit and human sera it is of such magnitude that the optical density at 565 mμ was found to be related directly to the albumin concentration.

4. All pathological human sera with low albumin concentrations studied showed that their density values agreed with the values that were found for the same concentration of normal serum albumin.

5. No adsorption effects were observed when serum was added

to 0.005 N sodium hydroxide solution or to a M/15 phosphate buffer mixture of pH 11.2.

6. A comparison of the phosphate and the veronal buffers indicated that the buffer system has an influence on the magnitude of the density values and on the position of maximum absorption.

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URINARY HISTIDINE

DETERMINATION OF HISTIDINE IN URINE. HISTIDINE IN NORMAL AND IN PREGNANCY URINES

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Determination of Histidine in Urine

In connection with studies of histidine and carnosine, and in particular of a colored trimolecular complex of histidine, creatinine, and 2.5-dinitrobenzoate, it became desirable to determine histidine in urine. With the published procedures of Kapeller-Adler for the colorimetric estimation of histidine (1) and also of carnosine after hydrolysis to histidine, no unusual difficulty was anticipated in making the desired determinations. But in application of the procedure to urine, or even to pure histidine solutions, such wide variations in the analytical results were observed that confidence in them was destroyed.

The color reaction involves bromination of histidine in acid solution, followed by neutralization of the acid with excess of ammonia, and heating of the alkaline solution, whereupon a blue or violet color develops. The reaction was discovered by Knoop (2), who considered that it was useful only as a qualitative test. Hunter (3) attempted to stabilize the color by extracting excess bromine with chloroform, and Kapeller-Adler showed that if precipitation of the colored substance could be avoided, histidine could be estimated quantitatively from the solution obtained. The only substances other than histidine which have been found to give the reaction are histamine, which gives a yellow, and methylhistidine (α-amino-β-methylimidazolepropionic acid), which gives a pale red of about one-fifth the intensity given by histidine. Since these substances seldom occur in appreciable amounts in solutions which contain histidine, it is usually assumed that the test is specific for histidine.

The chemistry of the changes of the histidine is not known; Hunter believes that 3 atoms of bromine are involved, and Kapeller-Adler gives evidence that both the primary amine and imide groups of histidine are necessary for color formation.[1] Chemical studies, as well as the attempted clinical applications of the reaction, are reviewed by Tschopp and Tschopp (4) and others (5–7). It is unfortunate that analyses, wherever given, are for histidine in mg. per cent of urine without other data to aid in interpreting their meaning.

Several modifications of procedure for the analysis of histidine are hereby recommended as contributing greatly to the dependability without adding to the complexity of the analyses. The suppression of color formation when histidine is added to urine is shown to be caused by urea, in addition to amino acids, which are known to have this effect (8). Histidine may be treated, as will be described later for urine, with a loss of less than 5 per cent, and it is concluded that the histidine of urine is subject to no greater loss.

Carbon tetrachloride containing 1 per cent (by volume) of bromine is aspirated by use of a current of air into the histidine solution in dim light. A uniform excess of bromine thus is maintained, and the solution volume change is not appreciable. After 20 minutes of aspirating, the bromine remaining with the histidine is removed by addition of phenol, which reacts instantly, and does not interfere with subsequent development of color. Conrad and Berg (9) used for this purpose 1 drop of saturated As_2O_3 in 10 per cent NH_4OH, which is not suited to our procedure. It is desirable that the color be developed in acid (pH about 4.6) rather than in ammoniacal solution (3, 6). The formation of a precipitate of phosphate is avoided, and the colors may be compared directly. The sensitivity of the reaction permits the accurate estimation of as little as 0.04 mg. of histidine per cc. of urine, and of smaller amounts with fair accuracy. Details of the procedure follow.

Reagents Required—
Sulfuric acid, 10 per cent by weight.

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[1] In this laboratory, Marjory Taylor has found that, after bromination of histidine, two primary amine groups are present, and that after heating only one remains. It may be inferred that the imidazole ring is opened by the addition of bromine, and that a different ring is formed during the heating.

Powdered $KMnO_4$.

Acid-washed decolorizing carbon.

Bromine in carbon tetrachloride. 1 cc. of bromine is added to 100 cc. of carbon tetrachloride.

0.5 per cent phenol.

Saturated sodium acetate in water (room temperature).

Histidine Standard A, 1 cc. = 1 mg. of histidine. Dissolve 123.5 mg. of histidine monohydrochloride in water and dilute to 100 cc. Add a few drops of chloroform.

Histidine Standard B, 6 cc. = 1 mg. of histidine. Pipette 50 cc. of histidine Standard A into a dry 500 cc. flask; add exactly 200 cc. of water and 50 cc. of 10 per cent H_2SO_4.

Analysis of Urine for Histidine

To 25 cc. of urine[2] are added exactly 5 cc. of 10 per cent sulfuric acid. Powdered $KMnO_4$ is now added in small portions with stirring until the purple color persists for about 15 seconds. Usually, with undiluted urine, about 75 mg. of $KMnO_4$ are required. Acid-washed charcoal (0.5 gm. of less) is then added; the solution is stirred for about 1 minute, and is then filtered. 6 cc. of the nearly colorless filtrate are pipetted into each of two test-tubes graduated at 10 cc. and 6 cc. (= 1 mg.) of the histidine Standard B into another. To one of the tubes containing the filtrate is added 0.4 cc. (0.4 mg.) of histidine Standard A. The tubes are now protected against exposure to light (standing in a comparator block and covered with a dark cloth), and into the solutions is aspirated bromine in carbon tetrachloride (about 1.5 cc. for each sample) at such a rate that a brown color of bromine is present. After 20 minutes, 1 cc. of 0.5 per cent phenol is added to each tube, and the bromination is stopped at once. The colorless, bromine-free solution, if kept cold, is stable for at least 3 hours. To each tube is added 1 cc. of saturated sodium acetate; they are shaken, and immediately are immersed in a boiling water

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[2] With pregnancy urines the colors obtained are usually too intense to be compared with the standard. The following volumes of urine are diluted to 25 cc. for analysis: urines of specific gravity 1.00 to 1.005, 25 cc.; 1.005 to 1.010, 20 cc.; 1.010 to 1.020, 15 cc.; 1.020 to 1.028, 10 cc.; 1.028 and higher, 5 cc. Proportionately smaller amounts of $KMnO_4$ and charcoal are required to yield nearly colorless filtrates. With dilute urines the effect of urine substances in suppressing the color often may be ignored.

bath for 1 minute (light excluded). The solutions are cooled to room temperature in an ice bath, diluted to 10 cc., and the colors are compared within a few minutes. Calculations are made as usual to give the histidine content per 5 cc. of urine. A correction must be applied, based on the recovery of the added histidine; e.g., 6 cc. of filtrate analyzed → 1.09 mg. of histidine, 6 cc. of filtrate + 0.4 mg. → 1.37 mg. of histidine, 1.37 − 1.09 = 0.28 mg. = 70 per cent of the 0.4 mg. added, 1.09 ÷ 0.70 = 1.56 mg. of histidine in 5 cc. of urine.

When amounts of histidine larger than could be matched with 1 mg. were present, the determination was repeated with diluted urine. More dilute standards were often required.

Indicator Color Standards for Histidine (Bromination in Dark, Aspiration with Air)

The reaction between histidine and bromine is affected by so many different environmental factors that, before the colors produced could be called maximal and constant, it was essential to prepare standard solutions of indicators to match the colors obtained with stated amounts of histidine. Standards have been prepared by mixing suitable amounts of three indicator solutions prepared according to the directions given by Clark (10). The proportions are given in Table I. When solutions of pure histidine give paler colors than are present in these mixtures, either incomplete color development is being obtained or fading has occurred. Since the indicator colors are not quite identical with those given by histidine, the use of a Wratten B filter (transmits 480 to 625 mμ, 85 per cent at 520 mμ) (Eastman Kodak Company) is recommended for matching in the colorimeter. The diluted color standards, unless stored in the dark and at low temperature, should be prepared fresh every 2 or 3 days, as the methyl red fades.

Relation of Color to Concentration of Histidine

Different known amounts of histidine were diluted to 5 cc. with water, 1 cc. of 10 per cent sulfuric acid was added, and bromination and color development were carried through as usual. The data (not shown here) showed that, within the usual limits of colorimetry, the color was directly proportional to the histidine present. After the color was developed, dilution with an equal volume of

water could be made without alteration of the color quality, or increase in the rate of fading.

Effect of Urea upon Color

During studies of the histidine content of urine, a test showed that urea lessened the amount of color produced. A suppression of color occurred also when urea was added to histidine after it had been brominated and the excess bromine had been removed, but once the color was developed, the effect was no longer ob-

TABLE I

Color Standards for Histidine

The stock indicator solution consists of bromocresol green, 0.04 per cent, 5 cc.; methyl red, 0.02 per cent, 7 cc.; phenol red, 0.02 per cent, 1 cc. Dilute the mixture to 50 cc. with phthalate buffer, pH 4.6.*

Conditions	Indicator A	Phthalate buffer	0.1 N acid or base	Water	Histidine equivalent
	cc.	cc.	cc.	cc.	mg.
Air	6	8	0.7 NaOH	1.3	1.0
	3	5	0.1 HCl	3.0	0.8
	3	5	0.2 "	6.3	0.6
	3	5	0.2 "	14.3	0.4
CO_2 (followed by addition of phenol and aeration for 1 min.)	3	4	0.4 NaOH		1.0

With different indicator preparations slight variations in the amounts of NaOH or HCl, with converse changes in H_2O added, may be necessary.

* To 50 cc. of 0.2 M potassium hydrogen phthalate are added 9.72 cc. of 0.2 M NaOH, and the solution is diluted to 200 cc.

servable. The conclusion seemed inevitable that urea reacts with some brominated substance derived from histidine, or else with histidine itself. Various amounts of urea were added to solutions containing histidine, and the relation between urea content and color was determined. The data are reported in Table II.

The suppression of color seems to depend more upon the total amount of urea present than upon the relation of urea to histidine. Therefore, by adding a known amount of histidine to a urine filtrate and determining the inhibiting influence of the urine components, the histidine of the urine may be calculated without analysis for

urea. That the color is suppressed in urine was shown by removing the urea with urease; a typical analysis (one of several which were carried out) is cited. The histidine, measured in mg. per cc., was 0.116 for urine (unmodified), 0.094 for urine + 300 mg. of urea per 5 cc., 0.240 for urine with urea removed, and 0.176 for urine with urea removed and 300 mg. of urea added per 5 cc.

Observations upon Color

In order to observe the color produced after bromination of histidine *in the absence of air and light*, bromine was aspirated into acidified histidine solutions by use of a stream of carbon dioxide,

TABLE II

Effect of Urea upon Color Formed by Histidine

Bromination in the dark, with air, for 20 minutes. In each case, the volume before bromination was 6 cc.

Urea present			
50 mg.	100 mg.	150 mg.	200 mg.
Histidine determined			
mg.	mg.	mg.	mg.
0.78	0.67	0.59	0.55⎱ 1 mg. histidine present
0.76	0.65	0.58	0.57⎰
0.36	0.33	0.22	0.21⎱ 0.5 " " . "
0.36	0.35	0.23	0.22⎰

light being excluded. It was soon found that, for reasons which are at present unexplained, maximum color was not produced unless the solution was shaken with air after the free bromine was removed. Under these conditions, the color differed from that obtained when the bromine was aspirated with air, and matched the indicator standard given in Table I. The color was a mixture of red and blue, and the relative amounts of the red and blue components varied in unpredictable ways. The total color, however, when matched against an indicator standard by use of a Wratten C_4 filter (transmits 255 to 500 mμ, 20 per cent at 460 mμ), always was found to be proportional to the histidine content of the solution. The red component of the color was bleached instantly when the solution was made ammoniacal, whereas the

blue component remained, which indicates that the chemical reactions producing the two colors are different. After bleaching of the red, heating of the ammoniacal solution did not increase the amount of blue, nor did reacidifying restore the red. That an equilibrium between the red and blue colors existed was indicated by the fact that during the heating to develop the color the solution first was pink and then became blue as the heating was continued. At the end of 1 minute, it was deep blue, and upon being cooled, changed again to violet, owing to the reappearance of the red. These observations indicate that at least two reactions occur during bromination of histidine, and that accurate spectrophotometric studies of the two colors should be made to relate them to the chemical changes.

When bromination of histidine is effected by aspirating with air, the above color variations are not observed. The color is brighter red, and is far more reproducible in hue than when CO_2 is used.

Exposure to ordinary daylight, especially during the bromination under CO_2, has been found regularly to diminish the color obtained with histidine. For this reason, light has been excluded.

Histidine Content of Normal and Pregnancy Urines. Creatinine to Histidine Ratio

In an attempt to determine whether or not the histidine excretion may be used as an index of pregnancy, we have determined the specific gravity, creatinine, and histidine content of normal and pregnancy urines, and, after studying the data, have plotted the ratios of creatinine to histidine, each in mg. per cc. of urine, as being a convenient means of characterizing the urines with regard to their histidine content. In this way, the urines, regardless of their water content, should fall into either of two groups. If the histidine content is high, as in pregnancy, the urine would be characterized as having a low creatinine to histidine ratio as compared with that of the male or the non-pregnant female. Differentiation of urines in this way is based upon a convenient number scale, ranging usually between 1 and 20. Accordingly, we have analyzed urines collected without regard to time, to the diet, or to the physical condition of the subjects. One urine sample per individual was included, and no data have been ex-

cluded. The male donors (43 in number) were mostly medical and dental students, the non-pregnant females (61 in number) were medical students and nurses, and the pregnancy urines (107 in number) were obtained at the prenatal clinic of the Buffalo General Hospital, the cases being from 2 to 9 months from the onset of pregnancy. There was no relation between the months of pregnancy and the size of the ratio. The values are summarized in Fig. 1 as simple frequency distribution curves, so that both the frequency of the ratios and the spread can be seen at a glance. As most of the ratios in pregnancy were determined upon diluted
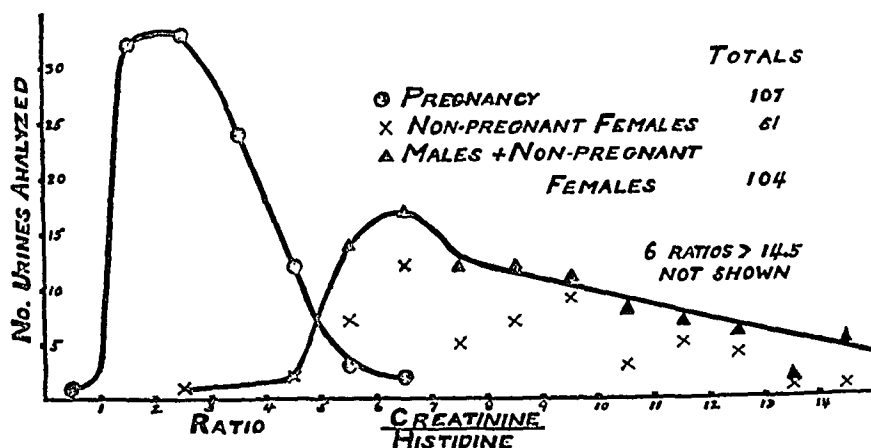
Fig. 1. Simple distribution curves for the ratio of creatinine to histidine for normal and pregnancy urines.

urines, and in most cases were below 4, corrections for the suppression of color by urine constituents were made only in the small number of cases in which the ratio was greater than 4, and those in which undiluted urine was used. A more accurately placed curve for the pregnancy urines would be slightly to the left of that given, except in the range 4 to 6. On the contrary, the ratios for the non-pregnancy urines were all corrected for the suppression of color, so that the position of this curve is essentially correct. The grouping of the ratios in pregnancy over the smaller numbers is in striking contrast to the wide range of the non-pregnancy ratios over the larger numbers. The ratios for pregnancy urines ranged from 0.9 to 6.4, with an average (107 determinations) of 2.71; for the non-pregnant female, with one exception, from

5.5 to 18, with an average (61 determinations) of 8.68; and for the male from 5 to 15.0, with an average (43 determinations) of 9.23. Although the averages of the ratios indicate a sharp separation between the types of urine analyzed, there is moderate overlapping of the distribution curves between the values 4 and 6. Therefore, the ratio, as determined upon casual urine samples, cannot be used as an unequivocal index as to pregnancy. Several males and non-pregnant females, whose urines gave unusually low ratios with a first sample, gave higher ratios when second samples were collected and analyzed several days later. Also, urines from several of the pregnant women showed diminished values for the ratio from about 5 at one time to about 4 at another. It seems from these observations that the extent of the overlapping of the distribution curves could be lessened somewhat by analyzing several specimens of urine per individual, and plotting average values of the ratios, whereby the usefulness of the data as indicating pregnancy would be increased correspondingly. It is clear that, if the creatinine-histidine ratio is to be useful as an indication of pregnancy, conditions must be found under which the ratio distribution curves are distinctly separated in the range of 4 to 6. From the data at hand, which apply only to normals and to pregnancies of 2 or more months duration, it is reasonable to estimate the probability of pregnancy as being very great for ratios smaller than 4, and the probability of pregnancy as being very slight when the ratio is greater than 6, but probability figures for pregnancy when the ratios are between 4 and 6 should be considered as having *no significance at all*. About 16 per cent of the urines studied fell within this range. Possibly more definite separation between pregnancy and non-pregnancy distribution curves might be obtained if the urines were collected under postabsorptive or basal conditions. It is not improbable also that figures representing the rate of histidine excretion after ingestion of a known amount of histidine would be more useful than a ratio of creatinine to histidine.

Comparison of the spread of values for the ratios for the pregnancy and non-pregnancy urines may seem to indicate that the non-pregnant group shows the more variable excretion of histidine. But the wide range probably occurs because the histidine excretion is small, so that small differences in histidine content of the

urine, or of errors in its estimation, are reflected as large differences in the ratios. Small changes in excretion of histidine might readily be of dietary origin. On the other hand, when larger amounts of histidine are excreted, as in pregnancy, small variations in histidine output change the ratio very little. Dietary factors alone can hardly account for these low ratios.

In order to be sure that the low ratios of pregnancy urines are not to be attributed to the excretion of creatine rather than of

TABLE III

Comparison of Ratios of Creatinine to Histidine and of Creatine + Creatinine to Histidine in Pregnancy Urine

Creatine is expressed as mg. of creatinine.

				Ratio	
Sp. gr.	Histidine	Creatinine	Creatine + creatinine	Creatinine / Histidine	Creatine + creatinine / Histidine
	mg. per cc.	*mg. per cc.*	*mg. per cc.*		
1.030	0.72	1.75	2.22	2.4	3.1
1.012	0.13	0.40	0.63	3.1	4.9
1.030	0.47	1.71	2.16	3.5	4.4
1.009	0.09	0.19	0.23	2.1	2.5
1.005	0.04	0.13	0.17	3.2	4.7
1.017	0.09	0.41	0.73	4.5	8.4
1.010	0.12	0.47	0.52	3.9	4.3
1.026	0.42	1.68	2.18	4.0	5.2
1.025	1.02	1.12	1.42	1.1	1.3
1.021	0.29	0.92	1.35	3.1	5.0
1.022	0.37	0.71	1.07	1.6	2.1
1.017	0.25	0.41	0.50	1.6	2.1
1.016	0.33	0.55	0.64	1.6	1.7

creatinine, analyses were made for creatine by the sulfuric acid procedure (11). The data are reported in Table III, and show beyond doubt that the excretion of creatine does not account for the low creatinine to histidine ratios.

SUMMARY

A procedure for the estimation of histidine in pure solutions and in urine by use of the Knoop bromination reaction is described.

Directions are given for the preparation of indicator standards by use of which the histidine colors may be evaluated.

Several peculiarities of the histidine colors are described.

The ratio of creatinine to histidine (each in mg. per cc.) has been determined for normal and pregnancy urines. During pregnancy, the ratios range from 0.9 to 6.4 with an average value of 2.71, and in non-pregnancy from 2.5 to 17.7 with an average value of about 8.9.

From a study of the simple distribution curves for the ratios, it is concluded that the estimation of histidine in relation to creatinine of the urine cannot be recommended at present as a reliable test for pregnancy, as about 16 per cent of the urines studied could not be definitely classified. The estimation may be recommended, however, as a valuable aid in the rapid diagnosis of, or routine testing for, pregnancy.

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THE CHEMICAL NATURE OF SCARLET FEVER TOXIN*

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There is considerable confusion in the literature regarding
scarlet fever toxin and "toxic or poisonous" substances obtainable
from cultures of scarlet fever streptococci. In 1934, for instance,
Hooker and Follensby (1) described an A and B toxin of scarlet
fever; in 1936 Kodama (2) separated three substances from strep-
tococci which gave skin reactions; and in 1938 Eaton (3) in a review
rallied to the opinion of the plurality of the toxin.

The substance under discussion here is the specific soluble toxin
of scarlet fever. There is no evidence thus far that more than one
such substance exists and in order to demonstrate its presence the
following tests should be made: (1) The substance should be
destroyed by boiling; (2) suitable dilutions injected into the skin
of numerous human subjects should give positive reactions when-
ever a positive reaction is given by one skin test dose of a standard
scarlet fever toxin obtained from streptococci which have produced
typical experimental scarlet fever in man, and give negative
reactions when the reaction to the standard toxin is negative; (3)
after giving a positive reaction in a human subject who gives a
positive test with standard scarlet fever toxin, it should, in proper
dilution, give a negative test in the same individual when mixed
with a suitable amount of specific scarlet fever antitoxin; (4)
it should produce nausea, vomiting, fever, malaise, and a typical
scarlet fever rash in susceptible human subjects when given
hypodermically in a single dose of 1000 to 3000 skin test doses.

The substance discussed here has been subjected to all of these

* Read before the meeting of the American Society for Clinical Investi-
gation at Atlantic City, May 6, 1940.

tests and is, therefore, specific scarlet fever toxin. It was prepared according to the method given by Dick and Boor (4). The toxin, a white, fluffy substance, dissolved readily in water, giving a yellow color in concentrated solutions, and contains from 20,000 to 30,000 skin test doses per mg.

From a study of the chemical properties of this purified toxin, as reported in this paper, the conclusion is drawn that scarlet fever toxin is a protein of small molecular weight, some amino groups of which are essential for its activity. Furthermore, a study of the electrophoretic mobility of the toxin at different hydrogen ion concentrations has shown that the toxin may be freed by electrophoresis of some nitrogenous impurities still present. In all the experiments the activity was tested with the skin test for susceptibility to scarlet fever as developed by Dick and Dick (5).

Effect of Temperature—It is generally agreed that scarlet fever toxin is very resistant to heat. To test the effect of temperature, the purified toxin (0.5 mg. per cc. dissolved in phosphate buffer of pH 7.4) was heated for 1 hour at different temperatures. Heating for 1 hour without shaking up to 90°, and for 45 minutes up to 100° (boiling water bath) caused no loss of activity. Bubbling either purified nitrogen or oxygen through the solution when heated in a boiling water bath resulted in loss of activity. Apparently, agitation at this temperature produced denaturation of the toxin as agitation at lower temperatures produces denaturation of proteins of higher molecular weight (for example, hemoglobin).

Effect of pH—It is generally believed that scarlet fever toxin is very unstable in alkaline solution, although no study of the influence of hydrogen ion concentration on the activity of the toxin has been reported. To test this influence, the universal buffer of Theorell and Stenhagen (6) was used from pH 2.08 to 11.77; by the use of this buffer, the chemical nature of the electrolytes was kept identical. The toxin solutions (1 mg. of toxin dissolved in 1 cc. of water plus 4 cc. of buffer) were kept for 24 hours at room temperature (±25°). They were then neutralized, diluted to the proper dilutions for skin tests, and filtered through Jena fritted glass filters for bacteriological filtration. The pH values of the solutions were measured by both the glass and hydrogen electrodes at 25°. As can be seen in Table I, the toxin was extremely re-

sistant to changes in the hydrogen ion concentration, the stability being greater in acid solutions than in alkaline solutions; it did not lose its activity after 24 hours in 0.1 N HCl (pH 1.08), but it was destroyed when kept in 0.1 N NaOH or in buffer solutions of a pH value of 11.77.

Effect of Ketene—Acetylation with ketene has proved to be useful in determining the importance of amino groups for biological activity. It is a highly specific acetylating agent for aqueous solutions of proteins, and several investigators (Herriott and Nor-

TABLE I

Effect of pH on Activity of Scarlet Fever Toxin

The toxin solution containing 1 mg. per cc. was kept for 24 hours at room temperature (+25°); buffer, universal of Theorell and Stenhagen.

pH	Size of skin reaction
	mm.
1.08	22 × 16 (faint)
2.08	28 × 36 (bright)
3.01	38 × 40 "
3.97	46 × 32 "
5.05	38 × 32 "
6.03	34 × 42 "
7.05	25 × 28 "
7.99	33 × 30 "
9.03	32 × 30 "
10.00	35 × 23 "
11.18	35 × 25 "
11.77	Negative

throp (7), Stern and White (8), Pappenheimer (9)) have shown that primary amino groups react most rapidly, while the OH groups of tyrosine are acetylated at a much slower rate. With glucosamine in aqueous solution, only the NH_2 groups react with ketene, forming N-acetyl glucosamine (Bergmann and Stern (10)), while the OH groups in carbohydrates resulting from hydrolysis of egg albumin are not acetylated (Neuberger (11)). Ketene destroys the activity of diphtheria toxin (Pappenheimer (9)) and of tetanus toxin (Velluz (12)), and, to some extent, the toxicity of gonococcus and meningococcus cells (Boor and Miller (13)). To test the effect of ketene on scarlet fever toxin, 50 mg.

of the toxin were dissolved in 10 cc. of 2 M acetate buffer of pH 5.06, put into a cellophane bag, and kept inside a 1 liter beaker containing the same acetate buffer to avoid changes in the hydrogen ion concentration inside the bag.   Ketene was generated in the apparatus devised by Herriott (14).[1]   The ketene vapor, before reaching the toxin solution, was purified by being passed through a flask cooled with ether and CO_2 snow; the gas was bubbled at a rate of about 4 cc. per minute and samples were withdrawn at different intervals (5, 10, 20, and 40 minutes).   The solutions were then dialyzed overnight in cellophane bags at 3° in running distilled water, made neutral, and tested at two dilutions, one 10

TABLE II

Effect of Ketene on Activity of Scarlet Fever Toxin

50 mg. were dissolved in 10 cc. of 2 M acetate buffer, pH 5.06.   Ketene was bubbled at the rate of 4 cc. per minute.

Time after ketene treatment	Size of skin reaction	
	1 × 8000 dilution	1 × 800 dilution
min.	*mm.*	*mm.*
Control	27 × 25	
5	Negative	20 × 28 (bright)
10	"	18 × 15 (medium bright)
20	"	10 × 10 " "
40	"	Negative

times stronger than the other.   The toxin used as control was treated identically, except for the ketene treatment (Table II). The toxin, at a dilution of 1 × 8000 was completely inactive at the end of 5 minutes.   There was some activity at a dilution of 1 × 800, but it was completely lost at the end of 40 minutes.   This loss of activity, after a short treatment with ketene, may be taken as an indication that ketene destroys by acetylation the primary amino groups essential for the activity of the toxin.

Effect of Nitrous Acid—The action of nitrous acid on amino groups has been known for a long time, and was used by Levene and Van Slyke (15) for the determination of amino acids.   As shown by Philpot and Small (16), nitrous acid resembles ketene in

first attacking the primary amino groups in the protein molecule. If ketene destroyed the activity of scarlet fever toxin by acetylation of amino groups, nitrous acid would also destroy it.   Such indeed was the case.   The experiments were performed at pH 4.07 in acetate buffer.   To 1.15 cc. of 1 N acetic acid were added 3.85 cc. of H_2O, 5 cc. of a toxin solution containing 4 mg. per cc., and 345 mg. of $NaNO_2$ (final concentration 0.5 M).   After standing half an hour at room temperature, the solution was neutralized, dialyzed overnight as indicated, and tested.   For control, a toxin solution was used in which the $NaNO_2$ was replaced by an equimolecular concentration of NaCl and the acetic acid replaced by acetate buffer with a final pH value of 4.06.   Treatment with

TABLE III

Effect of Nitrous Acid and Iodine on Activity of Scarlet Fever Toxin

Substance	pH	Time of action	Size of skin reaction
		hrs.	*mm.*
Control......	4.06		20 × 18 (bright)
Sodium nitrite (0.5 M).	4.07	0.5	Negative
Control........ . . .	7.02		30 × 32 (bright)
Iodine (0.002 N). ..	7.02	6.0	Negative
" (0.002 ").....	4.63	6.0	"
" (0.002 ")........	3.01	6.0	20 × 20 (bright)

nitrous acid destroyed the activity of the toxin completely, thus giving more support to the conclusion drawn from the experiments with ketene (Table III).

Effect of Iodine—Iodine may react with proteins either as an oxidizing agent, or as a substitution agent, its activity as an oxidizing agent being increased as the hydrogen ion concentration increases, while its activity as a substitution agent diminishes under the same conditions.   The effect of iodine (0.002 N) on scarlet fever toxin was studied at three pH values, 7.02, 4.63, and 3.01, the solutions being kept in the presence of iodine for 6 hours at 25°. At the end of this time the iodine was destroyed by the addition of cysteine, the solution was diluted, dialyzed overnight, and the skin tests performed (Table III).   The toxin was destroyed by iodine at pH values of 7.02 and 4.63, while at a pH value of 3.01 it still retained activity (control, 30 × 32 mm.; with iodine, 20 ×

20 mm.). Doubtless, this iodine inactivation is due to action of the reagent on the amino groups of the toxin more than to strict oxidizing action, for when the oxidizing power was increased by making the solution more acid there was no inactivation.

Effect of Porphyrindin and Other Oxidizing and Reducing Substances—Porphyrindin, a dye of highly positive oxidation-reduction potential (E'_0 at pH 7.0 = +0.57 volt) was recommended by

TABLE IV

Effect of Porphyrindin and Other Oxidizing and Reducing Substances on Activity of Scarlet Fever Toxin at pH 7.06

Substance	Time of action	Size of skin reaction
	hrs.	mm.
Control.................................		35 × 30 (bright)
Porphyrindin (0.001 M)................	0.5	Negative
Control.................................		30 × 30 (bright)
H_2O_2 (0.009 M)......................	4.5	28 × 13 "
$CuCl_2$ (0.00015 M)........	4.0	22 × 20 "
Oxidized glutathione (0.033 M).........	4.0	28 × 15 "
Iodoacetic acid (0.001 M)..:..........	4.0	35 × 30 "
Cysteine (0.023 M).....................	4.0	19 × 20 "
Glutathione (0.023 M).......	4.0	28 × 21 "
$Na_2S_2O_4$ (0.02 M)...................	7.0	20 × 23 "
H_2S (saturated)......................	5.0	28 × 20 "
$Pt + H_2$..............................	6.0	29 × 22 "
Iodoacetamide (0.01 M)................	4.0	35 × 30 "
Alloxan (0.05 M)........	5.0	28 × 25 "
Caffeine (0.01 M)......................	4.0	33 × 35 "
Sulfanilamide (0.005 M)................	0.1	35 × 25 (faint)
" ultraviolet irradiation (0.005 M).........................	0.1	30 × 20 "

Kuhn and Desnuelle (17) as a reagent for the rapid oxidation of —SH groups in the protein molecule. As such it has been extensively used by Greenstein (18). The effect of porphyrindin, kindly supplied by Dr. L. Hellerman, was studied at pH 7.06 by treating the toxin with the dye (final concentration, 0.001 M) for half an hour, the skin tests being performed after dialysis and proper dilution. Porphyrindin, under such conditions, destroyed the activity of the toxin (Table IV).

The inactivation of the toxin by porphyrindin made it necessary

to study the effect of substances which combine with either the —SH or —S—S— groups of proteins, inasmuch as the presence of these groups as the active constituents of certain hormones (insulin, du Vigneaud (19)), enzymes (urea, Perlzweig (20), papain, Hellerman and Perkins (21), cholinesterase, Nachmansohn and Lederer (22)), and toxins (snake venoms, Micheel and Emde (23), Binet, Weller, and Robillard (24)) has been recently established. Of the compounds known to react easily with either —SH or —S—S— groups under physiological conditions (at neutral reaction and temperatures not above 38°) the following were used: as reagents for —SH groups, $CuCl_2$ (0.00015 M), CH_2ICOOH (0.01 M), CH_2ICONH_2 (0.01 M), oxidized glutathione (0.023 M), alloxan (0.05 M), caffeine (0.01 M), H_2O_2 (0.009 M); as reagents for —S—S— groups, cysteine (0.02 M), glutathione (0.023 M), $Na_2S_2O_4$ (0.02 M), H_2S (saturated), and Pt black plus H_2 (Table IV). Since none of these substances had any effect on the activity of the toxin, it must be concluded that no active —SH or —S—S— groups are present. The inhibiting action of porphyrindin must therefore be attributed to destruction of some active group other than a sulfhydryl group. To elucidate this question, a number of substances containing primary amine groups (glycine, phenylalanine, histidine, tryptophane, tyrosine, tyramine, histamine, 1,4-diaminobutane, diethylamine) were treated with porphyrindin at pH 7.33, 25°, a ratio of 10 parts of amine to 1 of dye being used. With tyrosine and tyramine the reaction was immediate, as shown by prompt and complete reduction of the dye; with tryptophane, the dye was half reduced at the end of 1.75 hours. Porphyrindin was reduced by tyrosine and tyramine, presumably by oxidation of their OH groups. From these experiments it is suggested that tyrosine may be one of the active groups of the toxin, destroyed by ketene by action on its NH_2 group and nitrous acid, by porphyrindin and ketene by action on its OH group.

Carpenter and Barbour (25) reported that the toxins from *Staphylococcus aureus* and *Clostridium welchii* were inactivated by treatment with sulfanilamide. Neither sulfanilamide nor the oxidation product obtained by treatment with ultraviolet light had any effect on the activity of scarlet fever toxin (Table IV).

Effect of Pepsin and Trypsin—Contradictory reports have been published on the effect of trypsin on the activity of scarlet fever

toxin. Huntoon (26) states that the toxin is destroyed by trypsin, but Kodama (2) on preparing his toxin submitted the solution to the action of trypsin to hydrolyze the protein impurities. To test the effect of trypsin under optimum conditions for the activity of the enzyme, toxin solutions in 0.01 M Na_2HPO_4, containing 1 mg. per cc., were incubated at 38° with trypsin (0.13 mg. of active powder per cc. of toxin solution) for 3, 6, 9, 24, and 48 hours. At the end of the incubation period the solutions were neutralized with 0.01 M KH_2PO_4, diluted, and tested. When the incubation period was extended to 48 hours, more trypsin was added after 24 hours incubation.[2] In no case did trypsin destroy the activity

TABLE V

Effect of Pepsin and Trypsin on Activity of Scarlet Fever Toxin
Incubated at 38°.

Substance	pH	Time of action	Size of skin reaction
		hrs.	*mm.*
Control....................	3.01	12	35 × 25 (bright)
Pepsin....................	3.01	6	20 × 20 "
"	3.01	12	30 × 25 "
Control....................	3.01	24	15 × 10 (faint)
Pepsin....................	3.01	24	15 × 18 "
Control....................	8.5	24	35 × 30 (bright)
Trypsin....................	8.5	24	38 × 22 "

of the toxin. The same results were found when pepsin was used as the proteolytic enzyme. The experiments were performed at a pH value optimum for enzyme activity, namely 3.01 (Table V).

Isoelectric Point of Scarlet Fever Toxin—The electrophoretic mobility of the scarlet fever toxin was determined with Theorell's cataphoresis apparatus for test purposes (27) at 3.5° to avoid any danger of convection currents. The current intensity was kept at 5 milliamperes and the experiments terminated at the end of 3 hours. The toxin, dissolved in the buffer solution, was introduced in the U part of the apparatus, leaving five cells in both branches of the apparatus free for migration of the toxin. At the end of the experiment, the rubber disks were moved so as to

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[2] In these experiments, trypsin and pepsin solutions were previously tested for skin reactions and were found to give negative reactions.

separate the different cells; the fluid of the cells was pipetted off, and the nitrogen content determined. After neutralization, dilution, and filtration, the skin tests were performed with the contents of each cell. The electrophoretic mobility of the toxin was studied at five pH values (Table VI), 4.56, 5.24, 5.76, 6.08, 6.98. (The pH values quoted here are those calculated from measurements made at 25°, and by the use of the temperature coefficients given by Clark (28).) The toxin migrated towards

TABLE VI

Electrophoretic Mobility of Scarlet Fever Toxin As Determined with Theorell's Cataphoresis Apparatus

$T = 3.5°$, $+ =$ positive test for toxin activity, $- =$ negative test for toxin activity, $U =$ mg. per cent of N_2 remaining in the U part of the cataphoresis apparatus. The figures represent mg. per cent of N_2 in the cell.

pH	Anode						Cathode						U
	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Cell 6	
4.56	2.94	2.45	1.94	2.02	1.56	1.48	0.7	0.31	0.24	0.13	0.19	0.25	2.96
	+	+	+	+	+	+	−	−	−	−	−	−	−
5.24	1.48	0.33	0.25	0.25	0.25	?	2.0	1.48	1.38	0.62	0.25	0.25	3.1
	+	+	−	+	+	−	−	−	−	−	−	−	−
5.76	1.06	0.19	0.19	0.31	0.51	0.29	2.02	1.94	2.90	2.10	0.41		2.54
	+	−	−	−	−	−	+	+	+	+	+	−	
6.08	1.02	0.17	0.15	0.06	0.15	0.05	2.94	1.94	0.81	0.32	0.18	0.32	3.05
	+	+	−	−	−	−	+	+	+	−	−	−	
6.98	0.73	0.38	0.11	0.31	0.25	0.13	3.56	1.84	1.3	0.71	0.48	0.78	
	−	−	−	−	−	−	+	+	+	+	+	−	

the cathode from pH 5.76 on, and towards the anode from pH 5.24 to greater acidity. At pH 5.24, when the toxin migrated to the anode, some nitrogenous impurity migrated in the opposite direction, towards the cathode. At pH 5.24 and 4.56, when the toxin migrated to the anode, the average N content of the cells was 2.66 per cent, while the N content of the cells in the cathode branch, with no toxin, was 1.94 per cent. Electrophoresis of the toxin at pH 5.0 will therefore allow further purification of the toxin, for it will withdraw this nitrogenous impurity. Experiments in this direction are in progress.

The isoelectric point of the toxin, as determined by calculation of the ionic mobility from the data given in Table V was 5.55 (Fig. 1). This value is different from that given by Shinn (29), who reported it to be between pH 7.0 and 7.5; his experiments were carried out without proper control of the conditions necessary to perform electrophoretic studies, conditions studied carefully by Tiselius (30).

Ultrafiltration of Scarlet Fever Toxin—To estimate roughly the size of the scarlet fever toxin, the ultrafiltration of toxin solutions through graded collodion membranes was performed. The membranes were prepared according to the methods described by
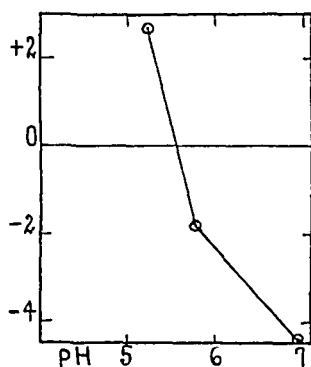
FIG. 1. Isoelectric point of scarlet fever toxin. Temperature, 3.5°. Ordinate, ionic mobility $\times$ 10⁵; abscissa, pH values.

Elford (31) and Bauer and Hughes (32) in a room kept at constant temperature and humidity (24° and 60 to 65 per cent relative humidity); the degree of humidity was diminished to that existing in the room for the preparation of membranes of small pore size. The membranes prepared from parlodion manufactured by the Mallinckrodt Chemical Works were similar in property and size of pores to those described by Bauer and Hughes. The average pore size of the membranes was calculated by an application of Poiseuille's law governing the passage of water through a capillary tube.[3] To test these membranes the following protein solutions of varied molecular weight were used: hemoglobin, prepared according to the method of Sidwell *et al.* (33), myoglobin, according

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[3] For the equations used to calculate the pore size of membranes, see Bauer and Hughes (32).

to the method of Theorell (34), cytochrome c,[4] according to the method of Keilin and Hartree (35), clupein, purchased from The British Drug Houses, Ltd. Filtrations were carried out under a pressure of about 780 mm. of Hg, the filter being connected to a nitrogen tank. The toxin filtered through all the membranes that let hemoglobin (mol. wt. 68,000), carbon monoxide myoglobin (mol. wt. 17,500), and cytochrome c (mol. wt. 13,000) pass through; moreover, it filtered through a cellophane membrane with a pore size less than 1 mμ. The toxin did not filter through

TABLE VII

Ultrafiltration of Scarlet Fever Toxin

The size of the control reaction was 40×35 mm., bright.

Average pore diameter	Hb, 68,000 mol. wt.	CO myoglobin, 17,500 mol. wt.	Cyto-chrome c, 13,000 mol. wt.	Clupein, ±2600 mol. wt.	Toxin, size of skin reaction
mμ					mm.
71.6	+++	+++	+++	+++	42×40 (bright)
39.2	+++	+++	+++	+++	35×30 "
16.3	+++	+++	+++	+++	36×28 "
8.4	+++	+++	+++	+++	30×22 "
3.1	0	+++	++	+++	28×20 "
2.1	0	0	+	+++	27×18 "
1.3	0	0	0	+++	20×25 (medium bright)
*	0	0	0	+++	30×20 (faint)
†	0	0	0	+++	Negative

* Cellophane from casings.
† Cellophane No. 600.

cellophane No. 600 although clupein (mol. wt. from 2000 to 4000) did (Table VII). We are well aware that the ultrafiltration method through collodion membranes, even if the membranes were of uniform pore size, can be applied only to molecules of spherical or globular configuration and to substances that are not adsorbed by the membrane. However, since the toxin passes through membranes that retain cytochrome c, while it does not pass through membranes which let clupein through, the evidence is strong in favor of the assumption that the scarlet fever toxin

[4] Care was taken to perform the ultrafiltrations at hydrogen ion concentrations optimum for non-adsorption by the membranes.

is a small protein with a molecular weight less than that of cytochrome *c* (13,000) and perhaps more than that of clupein (2000 to 4000). It may be recalled that the molecular weight of diphtheria toxin is about 72,000 (Lundgren, Pappenheimer, and Williams (36)) and that of crotoxin about 33,000 (Slotka and Fraenkel-Conrat (37)).

Some Components of Scarlet Fever Toxin—Dick and Boor (4) reported that the purified toxin used in these experiments contained 11.83 and 1.04 gm. per cent of nitrogen by formol titration, *i.e.* free amino nitrogen. Korschun *et al.* (38), who prepared a toxin by alcohol precipitation, reported a total nitrogen content
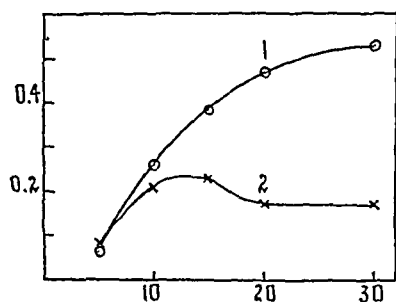
Fig. 2. Color intensity of toxin solutions with the orcinol method after warming at different times. Abscissa, time in minutes; ordinate, extinction measurement; Curve 1, readings with Filter S47; Curve 2, readings with Filter S53.

of 10.02 per cent and Stock (39) in a preliminary note reported a total nitrogen of 7 per cent. Undoubtedly, Stock was dealing with a substance not identical with the scarlet fever toxin as previously defined, since he found no free amino nitrogen, while the toxin as prepared by Dick and Boor (4), Korschun (38), and Veldee (40) does contain free amino nitrogen. As found by Korschun *et al.* (38), the toxin gave a red color with the biuret reaction, an indication that there is a peptide linkage and that the length of the peptide chain is small. It gave a positive Millon test, an indication of the presence of tyrosine in the molecule, and a faintly positive Hopkins-Cole test. It also gave a strongly positive Molisch reaction.

For the determination of the carbohydrate content, two methods were used: (1) titration with copper with the micromethod of

Somogyi (41) after previous digestion for 3 hours with 5 N H_2SO_4 (see Lyman and Barron for the details of the procedure (42)), and (2) the colorimetric orcinol method of Sørensen and Haugaard (43) as modified by Hewitt (44).   The carbohydrate content by the first method was 1.54 gm. per cent; by the second method, 1.43 gm. per cent.   On measuring the extinction coefficient of the blue solutions obtained after heating the toxin with the orcinol reagent at different intervals of time, the curve obtained with Filters S47 and S53 of the Zeiss Pulfrich photometer was similar to that reported by Sørensen (45) for mixtures containing 3 parts of mannose and 1 of galactose (Fig. 2).   The carbohydrate content of human serum is 3.2 per cent; that of horse serum, 2.4 per cent; of albumin. 1.08 per cent; of seroglycoid, 8.4 per cent; of ovomucoid, 20 per

TABLE VIII

Some Components of Scarlet Fever Toxin

Substance	Gm. per cent
Total nitrogen (Kjeldahl) .	11.33
Nitrogen (formol titration)...	1.04
Total carbohydrate (copper titration)	1.54
" " (orcinol, colorimetric)	1.43
Glucosamine (colorimetric).. .	0.73

cent (Hewitt (46)).   Thus the carbohydrate content of toxin is as low as that of proteins with the lowest carbohydrate content.

The glucosamine content of the scarlet fever toxin was also very low, 0.73 gm. per cent.   The toxin, dissolved in 4 N HCl, was subjected to 8 hours hydrolysis in a sealed tube in a boiling water bath.   After neutralization, the determination of glucosamine was performed in a Zeiss Pulfrich photometer with Filter S53 by the colorimetric method of Elson and Morgan (47) modified by Hewitt (46).   These low figures for carbohydrate and glucosamine content differ radically from Stock's preliminary report (39) on the erythrogenic toxin of *Streptococcus scarlatinae* in which it is stated that his substance contained large quantities of carbohydrates and glucosamine.

Since the toxin contained no phosphorus (no color reaction was obtained when the determination of total phosphates was attempted by the colorimetric method of Whitehorn (48)), it may be concluded that no nucleoproteins exist in the toxin (Table VIII).

From a study of the chemical properties of scarlet fever toxin described in this paper, namely the nitrogen content, the presence of free amino nitrogen, the resistance to high temperatures and to wide changes in the hydrogen ion concentration, the precipitation by high concentrations of ammonium sulfate, the color of the biuret reaction, and the passage by ultrafiltration through collodion membranes which do not let cytochrome c through, it is logical to conclude that the toxin is a protein of small molecular weight. The toxin is not only resistant to heat and to changes of pH value, but also to the action of active proteolytic enzymes such as pepsin and trypsin. The loss of activity of the toxin by brief treatment with ketene, nitrous acid, and iodine in neutral solutions is good evidence in favor of the theory that the activity is related to the presence of amino groups in the protein molecule, amino groups which are destroyed by these agents. As porphyrindin had been used for the estimation of certain mercaptans and of the sulfhydryl groups of certain proteins, the destruction of toxin activity by this reagent made it necessary to conduct a series of experiments with substances known to combine either with sulfhydryl or the disulfide groups. When none of these substances destroyed the activity of the toxin, it was found that porphyrindin was readily reduced by tyrosine, tyramine, and to some extent by tryptophane. Porphyrindin must therefore be rejected as a reagent for the detection of sulfhydryl groups of proteins, and the inactivation of the toxin by the dye may be attributed to its action on the OH group of tyrosine. (The positive Millon and Hopkins-Cole reactions in toxin are indicative of the presence of tyrosine and tryptophane in the toxin.)

The experiments on the electrophoretic mobility of the toxin are further evidence of its protein nature, for whether the toxin was made to migrate to the anode or to the cathode branch of the cell, a large portion of the nitrogen was retained in the active material. By assuming that the pure toxin contains 10 per cent nitrogen, it is calculated from these electrophoretic experiments that the material is 35 per cent pure. Since at pH values somewhat lower than those of the isoelectric point of the toxin (pH 5.55 at 3.5°) the protein impurity migrates towards the cathode

branch of the cataphoresis apparatus, there is here an easy method for further purification of the toxin.

The low carbohydrate and glucosamine content of the toxin, as well as the absence of phosphorus, speaks against considering it as a conjugated protein.

SUMMARY

Scarlet fever toxin is very resistant to the action of high temperatures (up to 100° for 15 minutes), wide variations in the hydrogen ion concentration (from pH 1.08 to 11.01), proteolytic enzymes (pepsin and trypsin), and a number of oxidizing and reducing agents. Its activity is destroyed by substances known to react with amino groups, such as ketene and nitrous acid. Evidence has been presented to support the view that inactivation produced by iodine and porphyrindin is also due to reaction with amino groups. From ultrafiltration with membranes of graded porosity it is concluded that the toxin is a protein of small molecular weight, between 13,000 and 4000. The isoelectric point of the toxin is 5.55 at 3.5°. As the carbohydrate and glucosamine content of the toxin is very low, and there is no phosphorus, it is concluded that the toxin is not a conjugated protein.

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THE CHANGES IN COMPOSITION OF SOLUTIONS OF CALCIUM CHLORIDE AND CALCIUM LACTATE IN THE INTESTINE*

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The work reported herein extends previous work dealing with the regulation and changes in the intestinal reaction and their effect on calcium absorption and subjects it to a detailed experimental analysis (5–7). The points of particular interest were (a) the effect of acidity on the absorption of calcium, (b) the effect of concentration on the absorption of calcium, (c) the mechanism of the control of intestinal reaction, and (d) the basis of calcium chloride acidosis. It appears that acidity alone does not increase calcium absorption. Other things being equal, more calcium is absorbed from concentrated than dilute solutions. The pH of alkaline solutions is quickly lowered below the initial point, partly by an influx of carbon dioxide. The rapidity of the adjustment of acid values depends upon the buffer strength of the solution involved. Water and salts pass in either direction through the intestinal wall to alter the osmotic pressure of the intestinal contents (3, 4, 9).

Methods

All experiments have been performed on dogs with Thiry-Vella fistulae. All of the intestinal loops were motile and apparently well nourished throughout the course of the work, and no change in absorptive activity was apparent. To all appearances the sections of the intestine had a normal blood and nerve supply and

were in an environment normal except for the absence of frequent passages of the products of digested food and secretions from the intestine above them.

The procedure finally adopted after many variations had been tried was as follows: After preparation, the loop of the gut was washed out daily with normal saline solution for about 2 weeks or until healing was complete. During an experiment the animal was confined on its back in a V-shaped animal stand. A perforated leather belt, passed about the body, held in place in the openings of the fistula 1-holed tapered stoppers through which passed catheters by which fluid entered and left the loop. This device effectually sealed the openings of the fistula and prevented loss of fluid by leakage. The catheters were joined in a closed circuit to a du Noüy pump, a condom, and a series of glass sampling tubes each having a volume of 12 cc. Thus, fluid could be circulated through the loop without changing its pressure. The condom served as a reservoir which, lying flat on a table, permitted change in volume of the fluid without significant change in the hydrostatic pressure of the system. The sampling tubes could be removed from the circuit and the volume of the solution altered, by absorption or secretion, without affecting the hydrostatic pressure in the loop, the latter being reduced to a minimum since the whole system rested in a horizontal plane on a table held at the level of the loop.

The results herewith reported were secured with four dogs, two having fistulae in the jejunum and two in the lower ileum.

Solutions of a single salt, either calcium chloride or calcium lactate, were circulated through the loop. For one series of experiments alkaline solutions adjusted to a pH of 7.0 to 7.5 with calcium hydroxide were used. For another series, acid solutions were used whose reactions were adjusted to pH 4.1 to 5.5 by means of the appropriate acid, either hydrochloric or lactic. In each of the series there were three concentrations, one hypotonic, in which the freezing point .varied from $-0.237°$ to $-0.488°$, and another hypertonic, with a freezing point of 0.680–$0.790°$ below 0; the third, the isotonic solution, was adjusted presumably as nearly as possible to freeze at $-0.571°$, but actually the freezing points varied from 0.520–$0.580°$ below 0. The calcium content of the solution varied from 99 to 178 milliequivalents

per liter for the hypotonic, 175 to 224 for the isotonic, and 310 to 499 for the hypertonic solutions. Each experiment continued for 3 hours.

pH was measured by the colorimetric method, the quinhydrone electrode, or the glass electrode, whichever was more convenient. Calcium was determined by precipitation as the oxalate and titration with permanganate and the amount of lactate by the method of Friedemann and Kendall (2). Total base was determined by the benzidine sulfate method and chloride by Van Slyke's modification of Volhard's method (8).

Results

Changes in Water Content—The changes in water contents and the freezing point depressions in experiments with two dogs are illustrated in Table I. In each instance two determinations are given to indicate the range in values obtained. The figures represent the actual volume of solution introduced into the loops, the volume remaining at the end of the experiment, and the difference calculated in cc. per liter of the original solution.

In general, hypotonic solutions lost water and hypertonic solutions gained water. Marked changes in water content of isotonic solutions were associated with concomitant changes in salt content, as is illustrated by the figures in Table II. These figures are taken from two experiments selected because the changes in freezing point depression are identical although the variations in salt and water content are pronounced.

Changes in Cation and Anion Concentrations

The observed changes in concentration were due, of course, to two factors, gain or loss of (1) water and of (2) solutes. The results have been analyzed from this standpoint, as illustrated by Table III. Similar calculations gave the corresponding values for the other constituents. Because carbon dioxide and cation other than calcium were present in the original solution only in small amounts, if at all, the changes in those ingredients due to dilution were usually nil. Absorption of water from the gut would, of course, result in an increase in concentration of solutes, giving the appearance of their secretion or diffusion into the loop.

In Chart I are shown the averages of approximately 70 experi-

TABLE I

Changes in Water Content and Freezing Point Depression of Solutions Remaining in Loops for 8 Hours

	Dog 1						Dog 2					
	Change in water			Change in freezing point depression			Change in water			Change in freezing point depression		
	Original volume	Final volume	Difference	Original solution	After 180 min.	Difference	Original volume	Final volume	Difference	Original solution	After 180 min.	Difference
	cc.	cc.	cc. per l.	°C.	°C.	°C.	cc.	cc.	cc. per l.	°C.	°C.	°C.
Hypertonic alkaline chloride	182	203	115.4	-0.790	-0.695	+0.095	184	211	146.6	-0.680	-0.585	+0.095
	204	224	98.2	-0.790	-0.690	+0.100						
Isotonic alkaline chloride	205	206	4.8	-0.520	-0.530	-0.010	221	274	239.6	-0.580	-0.570	+0.010
	184	185	5.4	-0.520	-0.510	+0.010						
Hypotonic alkaline chloride	174	162	-68.9	-0.380	-0.430	-0.050	158	216	367.0	-0.580	-0.620	-0.040
	189	170	-96.0	-0.380	-0.438	-0.058						
Hypertonic acid chloride	258	276	69.8	-0.789	-0.684	+0.105	168	224	333.3	-0.338	-0.495	-0.157
	159	168	56.6	-0.790	-0.695	+0.095						
Isotonic acid chloride	161	167	37.3	-0.525	-0.541	-0.016	208	160	-230.7	-0.338	-0.488	-0.150
	201	207	29.8	-0.522	-0.537	-0.015						
Hypotonic acid chloride	127	119	-63.0	-0.237	-0.390	-0.153	188	118	-372.3			
	144	131	-90.2	-0.237	-0.389	-0.152						
Hypertonic alkaline lactate	110	120	91.0	-0.780	-0.596	+0.184	221	327	479.8	-0.680	-0.565	+0.115
	109	120	101.0	-0.780	-0.610	+0.170						
Isotonic alkaline lactate	104	117	125.0	-0.540	-0.581	-0.041	183	233	273.2	-0.680	-0.572	+0.108
	110	125	136.4	-0.540	-0.605	-0.065						
Hypotonic alkaline lactate	138	134	-29.0	-0.322	-0.515	-0.193	196	250	275.5	-0.575	-0.540	+0.035
	127	116	-86.6	-0.322	-0.486	-0.164						
Hypertonic acid lactate	104	115	105.8	-0.784	-0.680	+0.104	166	217	307.3	-0.580	-0.578	+0.002
	107	115	93.5	-0.784	-0.600	+0.184						
Isotonic acid lactate	128	119	-70.3	-0.565	-0.572	-0.007	199	168	-155.7	-0.346	-0.502	-0.156
	126	138	95.3	-0.565	-0.596	-0.031						
Hypotonic acid lactate	121	121	-32.0	-0.345	-0.449	-0.104	225	209	-71.1	-0.340	-0.498	-0.158
	129	115	-118.6	-0.345	-0.427	-0.082						

ments on four dogs with calcium chloride solutions and 50 experiments on three dogs with calcium lactate solutions. There were

TABLE II

Changes in Composition of Two Isotonic Solutions of CaCl₂ during 3 Hours in Loop

	Concentration		Difference		
	Initial	Final	Found	Calculated*	Calculated†
Dog 1					
Volume, cc.	1000	1005	5		
Freezing point depression, °C.	−0.520	−0.510	+0.010		
Total cation other than Ca, m.eq. per l.	6.3	18.9	+12.6	−0.1	+12.7
Ca, m.eq. per l.	235.3	214.0	−21.3	−1.2	−20.1
Sum, " " "	241.6	232.9	−8.7	−1.3	−7.4
Cl, " " "	211.0	209.8	−1.2	−1.1	−0.1
CO₂, " " "	1.4	5.5	+4.1	0.0	+4.1
Sum, " " "	212.4	215.3	+2.9	−1.1	+4.0
Dog 2					
Volume, cc.	1000	1367	367		
Freezing point depression, °C.	−0.580	−0.570	+0.010		
Total cation other than Ca, m.eq. per l.	13.0	57.6	+44.6	−3.5	+48.1
Ca, m.eq. per l.	265.3	179.0	−86.3	−71.2	−15.1
Sum, " " "	278.3	236.6	−41.7	−74.7	+33.0
Cl, " " "	243.0	198.0	−45.0	−65.4	+25.4
CO₂, " " "	0.6	21.7	+21.1	−0.2	+21.3
Sum, " " "	243.6	219.7	−23.9	−65.6	+46.7

* Changes in original concentration with increase in volume due to water only.

† Changes in original concentration due to gain or loss of solute.

variations of considerable magnitude among the individual determinations but the averages as shown indicate clearly the qualitative trend in the results.

In the calcium chloride solutions the consistent and outstanding changes were (1) the absorption of calcium (usually in quantities which were greater, the greater the concentration or the acidity of the original solution), (2) the secretion of cation other than calcium in amounts approximately equal to or greater than the calcium absorbed, (3) the secretion of bicarbonate in quantities less than the cation, and (4) secretion or absorption of chloride (depending roughly on the concentration of the original solutions) in amounts usually insignificant in magnitude but always smaller than the amounts of calcium involved.   From   the   standpoint   of   the

TABLE III

Changes in Cation and Anion Concentrations

	cc.	cc.
Volume of solution introduced into system.............		182.0
" " " in system at end of experiment...	143.0	
Removed in samples................................	60.0	
Total final volume.................................		203.0
Change..		+21.0
	m.eq. per l.	*m.eq. per l.*
Calcium content of original solution.................	313.0	
Calculated final concentration if gain in fluid is due to H₂O......................................	281.0	
Difference due to dilution...........................		−32.0
Actual observed final concentration.................	297.8	
" " change...........................		−15.2
Difference due to gain of solute.....................		+16.8

acidosis-producing tendency of calcium chloride solutions these observations are important because they show that a preferential absorption of chloride ion does not occur and that an actual loss of cation does take place by secretion into the intestine and its replacement by calcium which may be promptly excreted in the urine.   The mechanism of calcium chloride acidosis thus appears to be a driving out of other cations by calcium.   For this explanation to be valid one must assume, of course, that the cation secreted into the intestine is excreted before it can be reabsorbed.

In the case of calcium lactate both calcium and lactate are absorbed, roughly in the order of the concentration of the original solutions.   Whereas more calcium than chloride was withdrawn

from the calcium chloride solutions, the opposite was apparent with the lactate solutions from which quantities of lactate greater than or at least equal to those of calcium were withdrawn. Cations other than calcium, chloride, and bicarbonate were all secreted into the intestine and in the case of the cation and chloride, in approximately constant and equivalent amounts irrespective of the
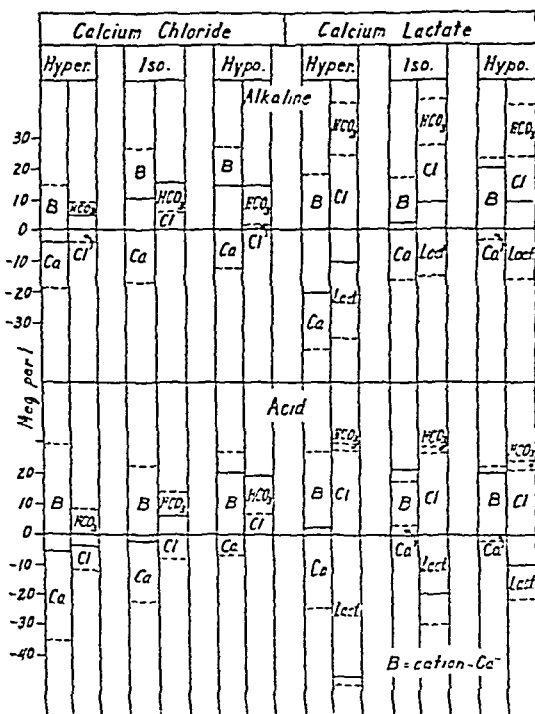
CHART I. Cation-anion changes in calcium chloride and calcium lactate solutions during 3 hours retention in loops.

concentrations and reactions of the solutions (1). Approximately equal quantities of bicarbonate were found in the three alkaline solutions, while almost none accumulated in the acid solutions whose reactions did not rise above about pH 5.3. The net effect of all these changes on the acid-base balance of the body would be small, since the absorbed calcium and lactate would be rapidly removed by excretion in the urine and combustion respectively,

while the loss of cation other than calcium into the intestine is about balanced by the loss of chloride.

Changes in Hydrogen Ion Concentration (Table IV)—It has previously been found that approximately isotonic solutions of calcium and sodium chlorides attain in a short time a fairly definite, constant reaction which is characteristic for different portions of the intestine (5). One would anticipate from that work that the contents of the sections of the gut used in the present studies would attain final reactions of about pH 6.8 and pH 7.2. Actually the mean values for all solutions except the acid lactates were pH 6.88 and 7.02 for the jejunum and ileum respectively, a difference which is statistically significant. The acid lactates were omitted because they apparently never reached a constant maxi-

TABLE IV

Average Values for pH Changes in 3 Hours

Type of solution	Acid				Alkaline					
	Chloride		Lactate		Chloride			Lactate		
	Initial	Final	Initial	Final	Initial	Minimum	Final	Initial	Minimum	Final
Hypertonic......	4.4	7.0	4.3	4.5	7.4	6.5	6.8	7.2	6.9	7.3
Isotonic..........	4.3	6.9	4.6	5.1	7.4	6.6	6.8	7.2	6.8	7.4
Hypotonic.......	4.3	6.9	4.5	5.3	7.5	6.5	6.9	7.1	6.7	7.2

mum. Their original reaction of pH 4.5 to 5.0 changed but little, presumably because these solutions were not far from the point of their maximum buffer capacity.

All alkaline solutions showed the remarkable phenomenon of a prompt decrease in pH to values in the acid range. In the case of the chloride this drop was considerable, going below pH 6.0 in some cases. This was followed by a slow return to higher figures, which, in the case of the lactates were frequently above the initial ones. As far as we know, this has never been observed before.

The initial step in the neutralization of alkali in the lactate solutions was an influx of carbon dioxide. This phenomenon was not apparent in the chloride solutions. Since in both types of solution there was a rapid decrease in pH, some other factors must be operative. At least a plausible explanation may be found in the

assumption that something in the gut, perhaps the secretion from the intestinal walls, reacts with calcium to precipitate or neutralize it. The subsequent slow adjustment that takes place is then brought about by the other agencies which produce similar pictures in acid solutions.

The fact that the intestinal contents tend to attain definite reactions which are characteristic for different levels of the intestinal tract is in itself proof that the intestinal contents do not reach the reaction of the blood, the approximate range of the reaction of the intestinal contents being from pH 6.5 to 8.0.

As has been pointed out before (7) the acidity of the intestinal contents is not the dominant factor in calcium absorption. With calcium chloride solutions more calcium was absorbed from the acid than from the alkaline solutions but the opposite was true for the lactates. This occurred in spite of the fact that the pH of the acid chlorides rose rapidly to approximate neutrality, while the lactates remained at about pH 5.0.

SUMMARY

Experiments are described in which solutions of calcium chloride and calcium lactate of known compositions were circulated for 3 hours through Thiry-Vella fistulae in dogs. They were analyzed at the end of the period. The changes in composition indicate the following conclusions.

Water and salts passed back and forth in either direction through the intestinal wall to bring the intestinal contents into approximate osmotic equilibrium with blood.

Concentrated, acid solutions of calcium chloride yielded the most calcium to the body. Acidity did not increase the absorption of calcium from lactate solutions, but the amount absorbed did increase with their concentration. Thus, the particular salt of calcium involved, as well as the acidity of the solution, is of importance in determining the amount of calcium absorbed.

Alkaline solutions quickly became acid. In lactate solutions this was accompanied in part at least by an inflow of carbon dioxide but some other mechanism was also involved. They were ultimately brought to the normal reaction of the section of the gut in which they were placed. The reaction of acid lactate solutions of pH 4 to 5 was only slightly altered during 3 hours.

The acidosis of calcium chloride is not due to the selective absorption of chloride ion from the intestine but is apparently caused by a displacement of other cation by calcium whose subsequent excretion produces a deficit in total cation.

We are indebted to the Department of Surgery of Vanderbilt University School of Medicine for the preparation of the intestinal loops.

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SOME OF THE OXIDATION-REDUCTION PROPERTIES OF THE CHORIONIC GONADOTROPIC HORMONE

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Gurin, Bachman, and Wilson (1–3) have recently made important contributions to our knowledge of the chemical nature of the gonadotropic hormone of pregnancy urine. They have given a method for the preparation of the hormone in a highly purified form and have described many of its outstanding chemical and physical properties. We have been especially interested in the reference of these authors to the unexplained, continuous, and apparently spontaneous inactivation of their highly purified material which takes place, particularly in aqueous solution, without detectable loss or rupture of certain portions of the molecule (3).

Earlier communications (4–6) have called attention to our observations relative to the oxidation and inactivation of a reducing factor of pregnancy urine which appears to be the chorionic gonadotropic hormone. Some of our more recent findings appear to shed light upon the observations of the above authors. The spontaneous oxidation of this reducing factor proceeds at a very slow rate but is more rapid in aqueous solution than in urine, where other stronger reducing agents tend to protect it. If activating agents are added and moderate heat is applied, the activity becomes of sufficient magnitude to bring about the reduction of dilute solutions of iodine or other oxidants. The object of this paper is to describe some of the rather unusual characteristics of this oxidation-reduction system.

EXPERIMENTAL

Preparation of Reducing Material—The experiments described in this report were undertaken some time ago and consequently

the procedure employed to separate the gonadotropic factor from pregnancy urine differs somewhat from that presented by Gurin, Bachman, and Wilson (2, 3); that is, Katzman and Doisy's method (7) was used with subsequent reprecipitation with acetone. Chilled protein-free urine of pregnant women was adjusted to pH 7.4 and the resulting precipitate was centrifuged and discarded. In this manner an appreciable amount of inert material was avoided at the outset. The urine was then acidified to pH 5 with glacial acetic acid and with vigorous stirring 75 cc. of acetone saturated with benzoic acid were added for each liter of urine. After the precipitate was centrifuged, it was washed three times with cold water saturated with benzoic acid and then with cold acetone to remove the benzoic acid. The acetone-insoluble fraction of the precipitate was repeatedly extracted with water at pH 7.4 and an equal volume of acetone was then added to the combined aqueous extract. The precipitate which formed at this point was centrifuged and discarded. After the pH of the acetone residue was adjusted to 5.0, the acetone concentration was increased to 70 per cent. The resulting insoluble fraction was centrifuged and again repeatedly extracted with water at pH 7.4. The fractional precipitation with 50 and 70 per cent acetone, detailed above, was carried out three times and the final precipitate was dried in a desiccator at 4°. The material so prepared assayed between 600 and 800 rat units per mg.

Potentiometric Studies—In addition to a marked gonadotropic effect, the material separated from pregnancy urine in the above manner invariably possessed characteristic reducing properties. In order to aid in identifying the reducing factor and to study some of its fundamental oxidation-reduction properties, it was titrated potentiometrically with iodine and potassium ferricyanide as oxidants.

In our earlier work potentiometric measurements were employed as a means of evaluating the efficiency of various methods of separating the pregnancy reducing factor from other urinary reducing substances (5). While these earlier studies aided in developing methods of separation, it became evident that the first titration curves were somewhat displaced from their correct position on the oxidation-reduction scale. This was apparently caused by a reaction at the platinum electrode in the presence of hy-

drogen. When the platinum electrodes were plated with gold, more correct potentials were observed.

The electrometric titrations were carried out in the usual manner. The solution to be titrated was placed in a conventional type of titration chamber to which the oxygen-free oxidant was introduced from a small sealed-in burette. In the work reported
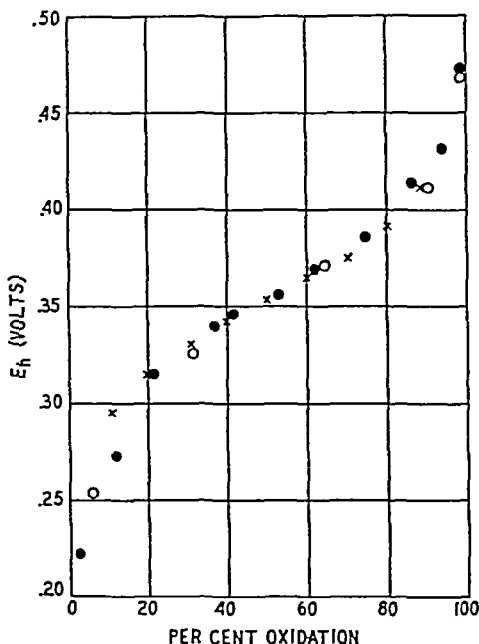
FIG. 1. Electrode potentials. ● represents the pregnancy reducing factor; ○ international standard gonadotropin; × the calculated values for an oxidation-reduction system which depends upon a single electron reaction.

here duplicate gold-plated platinum electrodes were employed and purified nitrogen was used to replace air in the apparatus. Before a titration was started, the entire sealed apparatus was repeatedly evacuated and flushed with nitrogen, with the solutions in place. The titration vessel was immersed in a water bath which was maintained at 38°. It was customary to titrate 5 mg. of the gonadotropic preparation dissolved in 10 cc. of M/15 Sørensen's phosphate buffer having a pH of 5.9.

The relation of electrode potential, E_h, to per cent oxidation is given in Fig. 1, which shows the potentials observed in the titration of the pregnancy reducing factor prepared as described above, the values observed in the titration of gonadotropin (international standard), and the calculated values for an oxidation-reduction system which depends upon a single electron reaction and which has an E_0 value equivalent to that represented by the mid-point in the titration curves. It appears that the same reducing agent is present in the two preparations and that the E_0 is $+0.354$ volt at $38°$. In view of these findings and the constant association of this potential reducing property with preparations of the chorionic

TABLE I

Relation between Reaction Rate and Buffer Salt Concentration

Buffer salt* added to 5 mg. of preparation	Time required to decolorize 5 cc. 0.0005 N iodine at 38°	
mg.		
0	18	hrs.
4.6	340	min.
9.3	120	"
18.6	8.5	"
27.9	5.0	"
37.3	4.25	"
46.4	4.0	"
92.9	4.0	"
139.3	4.0	"

* Prepared in a ratio of 9 moles of KH_2PO_4 to 1 mole of Na_2HPO_4 (pH 5.9).

gonadotropic hormone, and also since the urinary concentration of this factor has an unvarying relationship to pregnancy, it seems safe to conclude that the hormone itself or some substance always associated with it possesses reducing power. This is in agreement with Gurin, Bachman, and Wilson's indication (2) that their highly purified material slowly reduces ammoniacal silver nitrate in the cold.

Oxidation of Reducing Factor and Its Restoration—While many of the characteristics of this oxidation-reduction system are quite unusual, this fact in itself aids in differentiating the system from other reducing materials of biological origin. In aqueous solution it undergoes slow spontaneous oxidation. This oxidation can be

prevented by the addition of certain reductants. On the other hand, the oxidation is not greatly accelerated by the addition of strong oxidants unless the temperature is increased and phosphate buffer is present. The striking influence of the latter two factors will become apparent from an inspection of Tables I and II in which illustrative data are presented. The iodine titrations were made in the presence of 0.2 cc. of 2 per cent soluble starch (Merck) prepared according to Lintner. The reducing factor, buffer, and starch were adjusted to uniform volumes and heated to the designated temperature before the iodine, which was warmed to the same temperature, was added. From Table I it will be seen that with 5 mg. of the material in the absence of Sørensen's phosphate buffer 18 hours were required to reduce 5 cc. of 0.0005 N iodine

TABLE II

Relation of Temperature to Rate of Reaction

Temperature	Time required for 5 mg. of preparation to reduce 5 cc. 0.0005 N iodine*
°C.	min.
20	225
30	30
40	4
50	0.5

* In the presence of 50 mg. of Sørensen's phosphate buffer salt (pH 5.9).

at 38°. On the other hand, in the presence of phosphate buffer (pH 5.9) the reduction time was markedly reduced. It will be noted further that there is a limit above which further increase in phosphate has no added accelerating effect. The influence is presumably due to the phosphate ion, since with the substitution of sodium chloride or potassium chloride for the buffer (at the same pH, 5.9) no change in the reduction time was evident. From the data presented in Table II, it will be observed that in the presence of an optimum concentration of phosphate buffer the oxidation-reduction reaction is markedly accelerated by moderate increases in temperature.

Since a time factor is involved in the reaction and because the presence or absence of phosphate has such a marked effect upon the oxidation-reduction reaction, consideration was given to the

possible influence of other chemical agents employed in the titration. Potassium iodide is used to keep the iodine in solution. It was somewhat surprising to find that this salt retards the reaction rate. That this is true will become evident from an inspection of illustrative data given in Table III. Another surprising finding has been the effect of the soluble starch employed as an indicator. In the presence of starch the oxidation-reduction reaction is altered; and starch from several sources was found to behave differently. Thus, at equal concentrations, Kahlbaum soluble starch increases the rate of reduction about 3-fold over that observed with starch prepared according to Lintner, although neither

TABLE III

Retarding Action of Potassium Iodide

KI added to 5 mg. of preparation*	Time required to reduce 5 cc. 0.0005 N iodine at 38°
mg.	*min.*
0	4
0.14	5
0.28	8
0.56	12
1.12	24
1.68	24
2.0	24

* In the presence of 50 mg. of Sørensen's phosphate buffer salt (pH 5.9).

starch has any significant direct action upon the iodine. Just why such a difference exists is not entirely clear.

Although a more desirable preparation of soluble starch may be forthcoming, starch prepared according to Lintner is recommended, *because it appears to be uniform and is readily accessible.* In order to maintain a suitable and a uniform iodine solution which contains very little excess iodide the 0.0005 N iodine can be conveniently prepared by combining the following in order: 1 cc. of a stock potassium iodate solution (3.567 gm. of KIO_3 per liter of stock solution), 1 cc. of a stock potassium iodide solution (13.835 gm. of KI per liter of stock solution), 15 cc. of water, 0.5 cc. of 1 N sulfuric acid, and, after thorough mixing, sufficient water is added to provide a total volume of 200 cc.

The hormone tends to become biologically inactive when allowed to undergo spontaneous oxidation or following oxidation by means of strong oxidants; and the decline in biological activity parallels the decreased ability to reduce iodine under standardized conditions. A finding which appears to be of considerable interest and of practical importance is that the biological activity can be restored by treatment with certain strong reductants. That this is the case becomes evident from the results of the following illustrative experiments. The reducing factor was separated as described above. An aliquot of an aqueous solution assaying 12 rat units per cc. was heated, in the presence of phosphate buffer salt (pH 5.9), in a boiling water bath for 30 minutes. A relatively large surface was exposed to the air. After the solution was restored to its original volume, one-half was retained as a control and sufficient quinol was added to the other half to bring the concentration to 1 per cent.

10 per cent sodium hydroxide was added to the fraction containing quinol to bring the pH to approximately 8.5. After the material had stood for 30 minutes, the hormone was precipitated with 10 volumes of acetone at pH 5 and the precipitate was repeatedly washed with acetone. The dried material was dissolved in water and diluted to its original volume.

The heated control assayed more than 4 and less than 6 rat units per cc., while the "restored" fraction gave a slightly stronger reaction than the original unheated control when injected in equal amounts.

The reducing activity of the heated material as determined by iodine titration was a little less than one-half that of the original preparation, while the "restored" fraction was about 20 per cent stronger than the original preparation.

In another characteristic experiment the biological activity of the gonadotropic substance obtained from boiled pregnancy urine was increased more than 4-fold by reduction with quinol. Thus, 500 cc. of pregnancy urine were boiled for $1\frac{1}{2}$ hours in an open beaker. The original volume was restored and the gonadotropic factor obtained as described above. Part of the material so obtained was reduced with quinol and reprecipitated as described before. The material present in the non-reduced fraction was found to assay more than 125 and less than 250 rat units per liter

of the original urine, while the reduced fraction contained more than 1000 and less than 2000 rat units per liter of urine. The increase in reducing power as determined by iodine titration was similar in magnitude to the increase in biological activity.

That non-pregnancy specimens are relatively free of the reducing factor which is characteristic of pregnancy urine has been observed repeatedly. The protein-free specimens from a large group of normal adult males and young non-pregnant female subjects were compared with similar specimens from a comparable group of women in early pregnancy. The gonadotropic substance was extracted from the urine and then brought to its maximum reducing

TABLE IV

Relative Strength of Pregnancy and Non-Pregnancy Extracts

Specimen No.	Sp. gr.	Time required to reduce 5 cc. 0.0005 N iodine at 38° *
113-A. Non-pregnancy	1.029	More than 4 hrs.
113-B. "	1.028	" " 4 "
113-C. "	1.030	" " 4 "
113-D. "	1.025	" " 4 "
114-A. Pregnancy	1.028	8 min.
114-B. "	1.025	8.5 "
114-C. "	1.019	10 "
114-D. "	1.030	12 "

* In the presence of 1 cc. of 0.2 per cent soluble starch prepared according to Lintner.

power by treatment with quinol according to the procedures described above. The relative reducing capacity of each preparation was estimated by determining the time required for 5 cc. of 0.0005 N iodine to be reduced by the amount of material obtained from 50 cc. of urine. Typical results are given in Table IV.

DISCUSSION

The results of the potentiometric measurements and the constant association of a reducing property with chorionic gonadotropic preparations obtained from pregnancy urine or placental extracts strongly suggest that the reducing power can be attributed to the hormone itself.[1] The E_0 of this oxidation-reduction system

[1] Since this paper was prepared for publication, an opportunity has been afforded to study one of Gurin, Bachman, and Wilson's preparations

as well as the indication that the reaction depends upon a single electron exchange leads to the belief that the oxidation-reduction activity may be due to a bound metal ion or a derivative of tyrosine. Work now in progress points quite definitely to the hydroxyl group of a moderately labile derivative of tyrosine as the group which undergoes oxidation. If this is the case, it would appear to be in accord with the findings of Li, Simpson, and Evans (8) who have pointed out that the potency of human chorionic gonadotropin decreases as the phenolic hydroxyls of the tyrosine of the protein molecule are acetylated. It does not appear that the reducing action referred to here can be attributed to a carbohydrate group.

An account of further work in which the reducing capacity of the hormone was used as the basis of the chemical diagnosis of pregnancy will appear in a later paper.

SUMMARY

Studies of the oxidation-reduction characteristics of the chorionic gonadotropic hormone prepared by the benzoic acid method of Katzman and Doisy with subsequent reprecipitation with acetone have been made and may be summarized as follows:

1. Potentiometric studies upon the material so obtained and upon gonadotropin (international standard) lead to the conclusion that the oxidation-reduction properties can be attributed to the hormone itself. The E_0 of the system (at pH 5.9 and 38°) is $+0.354$ volt and the potential curve indicates 1 electron exchange.

2. The preparation undergoes slow spontaneous oxidation which can be inhibited by the addition of reductants and which is not accelerated appreciably by the addition of strong oxidants unless phosphate ion is present and moderate heat is applied.

3. The biological activity which has been decreased by oxidative changes can be restored by treatment with strong reductants.

The author wishes again to thank Dr. Henry Borsook of the California Institute of Technology for much valuable advice in relation to the electrometric studies, and also for facilities which were kindly made available for this phase of the work during the summers of 1937 and 1939.

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THE DEPOSITION OF LIPIDS IN VARIOUS PARTS OF THE CENTRAL NERVOUS SYSTEM OF THE DEVELOPING RAT*

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In previous studies from this laboratory it was shown that phospholipid activity of the brain (as judged by the rate of deposition of labeled phospholipid after the administration of radioactive phosphorus) varies with the age of the growing rat (1, 2). The highest phospholipid activity was found in the new born rat. From birth until the time the rat attains a weight of 50 gm. a decline in phospholipid activity occurs in the entire central nervous system. As growth proceeds beyond 50 gm., phospholipid activity decreases, but at a much slower rate than that observed between birth and 50 gm. Moreover, a dissimilarity was found in the activities of forebrain, cerebellum, medulla, and cord. Thus in the young rat the order of activity was cord, medulla, cerebellum, and forebrain, the cord being about twice as active as the forebrain. By the time the rat attains a weight of 200 gm. the activities in the various divisions of the central nervous system are about the same.

It seemed desirable at this time to determine the actual deposition of total phospholipid, cholesterol, and fatty acids in the central nervous system of the growing rat. Although Koch and Koch (3) have recorded the lipid differentiation for the whole brain of the rat, the deposition of lipids in its various parts, namely cord, medulla, cerebellum, and forebrain, has not been previously investigated.

The data recorded here were obtained from over 375 rats. Animals of the following weights were chosen for study: 5 gm. (new born), 15 gm. (1 week), 25 gm. (2 weeks), 30 gm., 50 gm., 100 gm., 200 gm., and 300 gm. Within each group the weights of the animals varied by not more than 5 per cent except in the case of the new-born and of rats weighing 300 gm., in which cases variations of 10 per cent were permitted.

The first three age groups were suckling at the time they were sacrificed for analyses. All others were maintained on the standardized stock diet.

Divisions of Brain—The manner in which the animals were sacrificed and the brains removed has been recorded elsewhere. The same divisions as those previously chosen for study with radioactive phosphorus were employed here; namely, forebrain, medulla, cerebellum, and spinal cord. Pools of these brain divisions were obtained from five to twenty animals; most of the pools from six rats. In the case of the rats weighing 5 and 15 gm., it was necessary to use several litters to obtain sufficient material for analysis. Each of the values recorded in Figs. 1 to 3 represents the average of six or more separate analyses, the results of which were in close agreement.

Extraction of Lipids—The tissue was extracted for 1 hour with 95 per cent alcohol at 55–60°. The alcohol was then decanted and the residue ground with clean sand. The ground residue was then subjected to two further extractions with 95 per cent alcohol, each for 2 hours. The material was then transferred to a Soxhlet apparatus and extracted first with ethyl ether for 6 to 8 hours and then with petroleum ether for 6 to 8 hours. The alcohol, ethyl ether, and petroleum ether extracts were combined and concentrated *in vacuo* at 55° to a volume of approximately 5 cc. in the presence of CO_2. This concentrate was extracted three times in separatory funnels with 30 cc. portions of petroleum ether and made up to a total volume of 100 cc.

Total fatty acids, phospholipids, and total cholesterol were determined in petroleum ether extracts. The oxidative procedures employed for the determination of these lipid constituents have been described elsewhere (4). Free cholesterol was measured on an acetone extract in which phospholipid had been completely pre-

cipitated. The details of this modification will be described elsewhere.

Results

Total Fatty Acids—The highest fatty acid content in the new born rat (5 gm.) was found in the cord and the lowest in the forebrain (Fig. 1). The former contained 2 per cent fatty acids, the latter 1 per cent. Very little increase in this lipid constituent occurred in the brain at the next stage examined; namely, rats weighing 15 gm. Maximum deposition of total fatty acids was
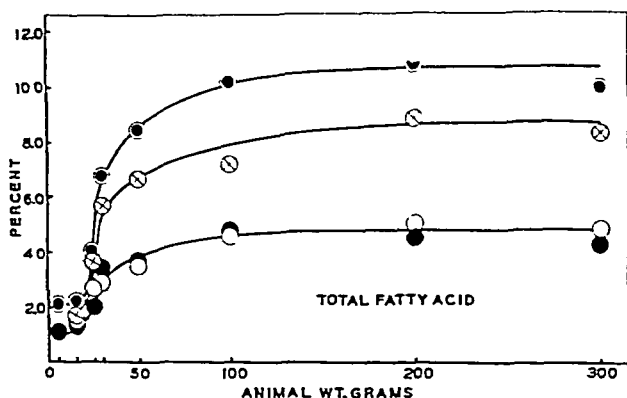
FIG. 1. The deposition of total fatty acids in the brain of the rat. ⊚ cord, ⊗ medula, ○ cerebellum, ● forebrain. In the 5 gm. rat (new born) the cerebellum and medulla were combined for analysis and contained 1.51 per cent fatty acids (average).

found between the intervals represented by rat weights of 15 (1 week old) and 30 gm. The total fatty acid content of the cord in the rat weighing 25 gm. (2 weeks) was twice that of the 5 gm. or new born rat; in the 50 gm. rat the content in the cord is double that in the 25 gm. animal. The brain continued to accumulate fatty acids until the animal attained a weight of 100 gm. Little, if any, change occurs thereafter in the percentage of this lipid constituent in any of the divisions of the central nervous system examined.

In the young as well as the adult rat, the fatty acids were not found uniformly distributed in the various parts of the central

nervous system.   A characteristic order was maintained through-
out: the cord contained the highest amount of fatty acids, the
medulla the next highest, the lowest amount being in the cere-
bellum and forebrain.   In the 200 gm. rat these differences are
quite marked: the cord contained 10.7 per cent fatty acids, the
medulla 8.5, the cerebellum 4.5, and the forebrain 4.0 per cent.
These differences in absolute amount are less striking in rats
weighing 5 to 30 gm., although in the new born rat the cord con-
tained twice as much fatty acid as the medulla.
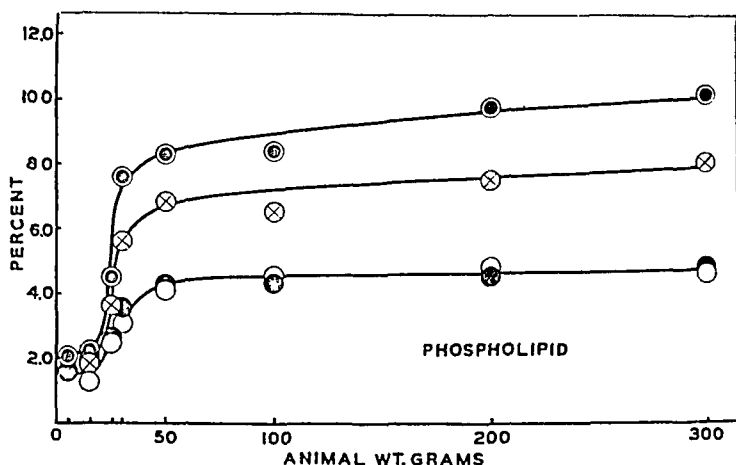
FIG. 2. The deposition of phospholipid in the brain of the rat. ◉ cord,
⊗ medulla, ○ cerebellum, ◉ forebrain.   In the 5 gm. rat (new born) the
cerebellum and medulla were combined for analysis and contained 1.76
per cent phospholipid (average).

Phospholipid—The rate of deposition of phospholipid in all
parts of the brain follows closely that of fatty acids (Fig. 2).
Little increase occurred between 5 and 15 gm., while the most
rapid deposition occurred between 15 and 30 gm.   The phospho-
lipid content in the spinal cord of the 50 gm. rat is twice that of
the 25 gm. rat and 4 times that of the 5 gm. rat.   The process of
accumulation leveled off after a rat weight of 50 gm. had been
attained.
    Marked differences in the phospholipid content of the various
brain divisions were found throughout all age groups studied.
Thus in the oldest animal studied the cord contained 10 per cent,
the medulla 8.0, the forebrain 4.5, and the cerebellum 4.5.   In

the 50 gm. rats, cord, medulla, cerebellum, and forebrain contained respectively 8.3, 6.8, 4.0, and 4.0 per cent.   The cord in the 1 week-old rat contained about twice as much phospholipid as the cerebellum.

Cholesterol—Significant amounts of esterified cholesterol were not found in the brain, an observation in agreement with previous findings (5). The results shown in Fig. 3 therefore represent free cholesterol. The characteristics observed for the accumulation of cholesterol in the brain were quite similar to those observed
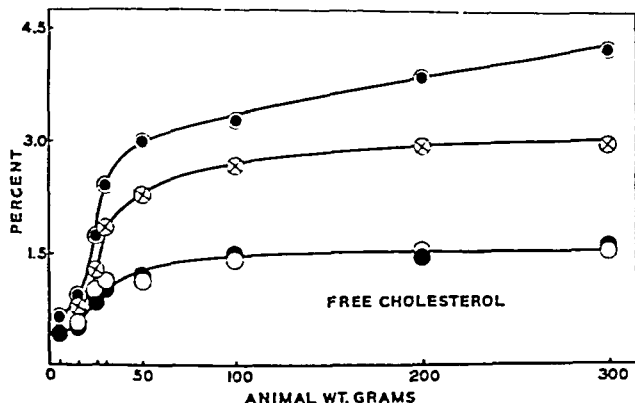
Fig. 3. The deposition of cholesterol in the brain of the rat. ⊙ cord, ⊗ medulla, ○ cerebellum, ● forebrain. In the 5 gm. rat (new born) the cerebellum and medulla were combined for analysis and contained 0.51 per cent free cholesterol (average).

for the other lipid constituents noted above. The most rapid deposition occurred between weights of 15 and 50 gm., the rate thereafter being greatly diminished. The cholesterol content of the spinal cord of the 25 gm. rat is about 2.5 times that of the 5 gm. rat, whereas the content of this lipid constituent in the 50 gm. rat is double that of the 25 gm. animal. The same order of content as found for total fatty acids and phospholipid was observed for free cholesterol. In the 300 gm. rat, 4.0, 3.0, 1.5, and 1.5 per cent cholesterol were present in the cord, medulla, cerebellum, and forebrain respectively. In the 50 gm. rat the parts contained 3.0, 2.2, 1.2, and 1.3 per cent respectively. In the 2 week-old rat (25 gm.) the cord contained twice as much cholesterol as the forebrain or cerebellum.

DISCUSSION

The present study shows that throughout the development of the brain after birth the lipid composition of its various parts is not uniform. At all times from birth on, the cord contained the highest amount of all lipid constituents. The next highest amounts were found in the medulla. Cerebellum and forebrain were almost identical in their lipid composition, particularly after a rat weight of 30 gm. had been attained, and throughout their entire development were markedly lower in their lipid content than either cord or medulla. This lack of uniformity observed

TABLE I

Ratio of Phospholipid and of Free Cholesterol to Total Fatty Acids in Brain

The arbitrary value 100 has been assigned throughout for total fatty acids and the other constituents are expressed as percentages thereof.

Animal weight	Cord		Medulla		Cerebellum		Forebrain	
	Phospholipid	Free cholesterol	Phospholipid	Free cholesterol	Phospholipid	Free cholesterol	Phospholipid	Free cholesterol
gm.								
5	94	30					137	36
15	100	42	107	41	85	38	140	37
25	109	42	99	35	90	37	125	41
30	111	35	98	32	106	39	100	29
50	98	35	102	34	117	38	115	33
100	82	32	91	37	98	30	98	32
200	91	36	86	33	97	30	101	32
300	102	43	98	36	97	32	112	37

here for the rat brain has been previously recorded for the human brain by McArthur and Doisy (6). These workers, however, obtained a different distribution. The brain stem was only slightly higher in lipid content than the forebrain, while the cerebellum showed a much lower content than either brain stem or forebrain. These differences in the various divisions may be related to the relative amounts of white and gray matter, and in this connection it is of interest to note that much higher amounts of lipids have been repeatedly observed in white than in gray matter. Randall (7) has also shown that the white matter from various areas of the cerebrum contains lipid similar to that found in the whole brain stem, a tissue known to be high in white matter.

The order of myelination in the brain follows the order of phylogenetic differentiation (8, 9). Thus the cord is the first to show evidence of myelination, the brain stem next, and the cerebellum and cerebrum last. It is of interest to note that the order of content of each of the lipid constituents studied here follows the above order of myelination, the highest content occurring in the division that myelinates first and the lowest in the part in which it occurs last.

Table I depicts the relation of the phospholipid and the cholesterol content of the brain to its total fatty acid content in the various age groups studied. The amount of total fatty acids throughout has been assigned the arbitrary value of 100 and the other constituents expressed as percentages thereof. The values shown in Table I suggest that the pattern by which the various lipid constituents are deposited is maintained relatively constant. Thus in the spinal cord of the 15 gm. rat the ratios of fatty acid to phospholipid to free cholesterol are 100:100:42, whereas in the 200 gm. rat these ratios are respectively 100:91:36. The medulla showed ratios of 100:107:41 in the 15 gm. rat and 100:86:33 in the 200 gm. rat. In the case of the forebrain, higher ratios were obtained for phospholipid in the earlier age groups, but the values for cholesterol were similar to those observed in the other brain divisions.

SUMMARY

1. The deposition of cholesterol, phospholipid, and total fatty acids was studied in various divisions of the central nervous system (spinal cord, medulla, cerebellum, and forebrain) of the rat from birth till the time it attained a weight of 300 gm.

2. In each part of the central nervous system, the maximum rate of deposition of these lipid constituents occurred during the time between birth and the time the rat attains a weight of 50 gm.

3. Differences in lipid composition in all parts of the central nervous system were found. Throughout the entire period of observation, cord contained the highest amounts of cholesterol, phospholipid, and total fatty acids. The medulla was next in order. The lipid composition of cerebellum and forebrain at all periods studied was almost identical, and their lipid content was always lower than in either spinal cord or medulla.

4. The pattern with respect to the amounts of cholesterol, phospholipid, and total fatty acids contained in each division of

the central nervous system from birth till full growth is maintained relatively constant.

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THE DETERMINATION OF PYRUVIC ACID IN BLOOD IN THE PRESENCE OF ACETOACETIC ACID

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The colorimetric methods for the determination of pyruvic acid (1–4) are based on the isolation of sodium pyruvate 2,4-dinitrophenylhydrazone and the reaction of the latter with strong alkali to form a red derivative. The corresponding hydrazones of acetone and acetoacetic acid also react in a similar manner with strong alkali (5). Case (1) reported that acetone is not measured in the determination of pyruvate. It has been found, however, as already reported by Lu (3), that with high concentrations of acetoacetic acid sufficient acetoacetate hydrazone remains intact to interfere with the accuracy of the determination. Thus precise measurement of pyruvic acid concentrations, when ketonemia exists, is not possible by present methods.

The Peters and Thompson modification (2) of the Case method (1) has been further modified by the writer (6) to avoid this source of error. Subsequently, improvements suggested by the more rapid methods of Lu (3) and Bueding and Wortis (4) have been incorporated into the procedure. Interference by acetoacetic acid is overcome by allowing the ethyl acetate extract to stand overnight. Tungstic acid is substituted for trichloroacetic acid as the protein precipitant to prevent decomposition of pyruvate hydrazone during the prolonged standing period in ethyl acetate. The increased acidity of the ethyl acetate extract when trichloroacetic acid is used causes a marked destruction of pyruvate hydrazone.

While the specificity of the method for pyruvate has been improved by the modifications introduced, it should be realized that the 2,4-dinitrophenylhydrazones of other α-keto acids

possibly present in blood may form red compounds with strong alkali and will also be determined as pyruvate. The 2,4-dinitrophenylhydrazones of oxaloacetic (5) and α-ketoglutaric acids have been found to react in this manner. "Pyruvate" is used therefore with the reservation that the substance measured may not be homogeneous.

Reagents—

27 per cent sodium iodoacetate solution. The pure salt is easily prepared from the acid (7). The solution is equivalent to 25 per cent iodoacetic acid solution.

$N/12$ sulfuric acid solution.

10 per cent sodium tungstate solution.

0.5 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. The acid is heated under a reflux condenser, the hydrazine is added, and the mixture is shaken until solution is complete. After reaching room temperature the solution is filtered.

Ethyl acetate, reagent quality.

1 per cent sodium carbonate solution. The anhydrous compound is used.

4 N sodium hydroxide solution. To prevent discoloration the solution is kept in a glass-stoppered Pyrex bottle.

Procedure

The pyruvate in the blood is first stabilized with iodoacetate (4). A tungstic acid filtrate is then made by the Folin-Wu-Haden procedure (8, 9). 10 cc. of the filtrate are transferred to a 60 cc. glass-stoppered Squibb separatory funnel and mixed with dinitrophenylhydrazine solution. 0.2 cc. of the latter is ample in the absence of ketonemia, 0.5 cc. for amounts up to 100 mg. per cent, and 1 cc. in the presence of 100 to 200 mg. per cent of acetoacetic acid. After 15 minutes, 1 minute extractions of the mixture are made with three successive 5 cc. portions of ethyl acetate. The combined ethyl acetate fractions are washed once with 2 cc. of distilled water and are then kept in the separatory funnel over another 2 cc. of water in the dark for 18 to 24 hours. After removal of the water the pyruvate hydrazone is transferred from the ethyl acetate to 1 per cent sodium carbonate solution by successive extractions with 2, 2, and 1 cc. portions. To prevent any loss of hydrazone from the stem of the separatory funnel,

between the first and second shakings the ethyl acetate is washed with 0.5 cc. of sodium carbonate solution. All four fractions are transferred to a separatory funnel. In order to remove any free hydrazine or interfering hydrazones the combined sodium carbonate fractions are now shaken with 1 cc. of ethyl acetate. The sodium carbonate extract is transferred to a 10 cc. glass-stoppered cylinder and the ethyl acetate remaining in the separatory funnel is washed with two 0.5 cc. portions of sodium carbonate solution which are also added to the contents of the cylinder. The volume of the alkaline extract is now made up to exactly 8 cc. with more sodium carbonate. Finally 2 cc. of 4 N sodium hydroxide solution

TABLE I

Recovery of Added Pyruvic Acid from Blood

Pyruvic acid added	Pyruvic acid recovered		Pyruvic acid added	Pyruvic acid recovered	
mg. per 100 cc.	*mg. per 100 cc.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
1.03	0.92	89.3	2.06	1.73	84.0
1.03	0.88	85.4	2.06	1.89	91.7
1.03	0.94	91.3	4.12	3.61	87.6
2.06	1.87	90.8	4.12	3.89	94.4

are added, the contents mixed, and the pyruvate estimation carried out in a photoelectric photometer.

Results

The recoveries of pyruvic acid from blood are shown in Table I. These results are better than those reported for the modified Peters-Thompson procedure (6) and are similar to those obtained by Wilkins, Weiss, and Taylor (10) who used the original Peters-Thompson method (2).

Determinations were carried out in the presence of varying amounts of acetoacetic acid added to the blood with and without the overnight period of standing in ethyl acetate. The acetoacetate was prepared according to the method of Shaffer (11) and the concentrations added were determined as bisulfite-binding substances (12). Recoveries of the β-keto acid as pyruvate by the two methods are reported in Table II. The blood pyruvic acid values in the absence of added acetoacetate revealed only

slight differences by the two procedures. When the determinations were made by the prompt extraction method in the presence of added acetoacetic acid, sufficient acetoacetate hydrazone remained to cause inaccurate pyruvate values, whereas with the method as described the interfering hydrazone was removed.

In these recovery experiments the bloods used were not stabilized with iodoacetate, but were pooled samples which had stood for several hours. Bueding and Wortis (4) have shown that on standing at room temperature the pyruvate level of blood decreases

TABLE II

Destruction of Acetoacetic Acid Dinitrophenylhydrazone after Addition of Acetoacetic Acid to Blood

The values are expressed as mg. per 100 cc.

Acetoacetic acid added	Recovery by prompt extraction method			Recovery by delayed extraction method		
	Ketonic acids as pyruvic acid	Pyruvic acid	Acetoacetic acid as pyruvic acid	Ketonic acids as pyruvic acid	Pyruvic acid	Acetoacetic acid as pyruvic acid
8	0.64	0.56	0.08	0.53	0.52	0.01
8	0.79	0.62	0.17	0.51	0.51	0.00
17	0.82	0.64	0.18	0.64	0.64	0.00
17	1.00	0.62	0.38	0.51	0.51	0.00
35	0.99	0.56	0.43	0.55	0.52	0.03
35	1.40	0.62	0.78	0.51	0.51	0.00
70	1.42	0.56	0.86	0.68	0.52	0.16
100	1.14	0.64	0.50	0.82	0.64	0.18
100	2.63	0.53*	2.10	0.60	0.53	0.07
200	5.00	0.53*	4.47	0.65	0.53	0.12

* The value which was obtained by the delayed extraction method was used.

in the absence of iodoacetate and increases in its presence. The use of iodoacetate was therefore omitted to avoid the possibility of interference in the recovery experiments.

The two procedures were also compared (Table III) with bloods containing normal and elevated concentrations of acetoacetic acid. The latter was determined by the bisulfite-binding method (12), the factor 28.05 being used to express total carbonyl compounds as acetoacetic acid in place of the 15.95 used for acetone. The normal range of acetoacetic acid is 1 to 3 mg. per cent. As demon-

strated in Table III with normal acetoacetate levels, the two procedures yielded similar pyruvate values. When ketonemia existed, the modified method described in this paper gave the lower and more correct figures. The difference can be attributed to the decomposition of residual acetoacetate hydrazone. The

TABLE III

Effect of Acetoacetic Acid on Determination of Pyruvic Acid in Blood

The values are expressed as mg. per 100 cc.

Patient No.	Apparent pyruvic acid by prompt extraction method	Pyruvic acid by delayed extraction method	Bisulfite-binding substances as acetoacetic acid
Normal patients			
1. Overnight fasting.	0.55	0.57	3.0
2. " "	0.85	0.85	2.8
3. " "	0.59	0.55	1.8
4. *140 hrs. fasting*..... ..	*1.12*	*0.83*	*16.6*
188 " "	1.00	0.96	7.4
1 hr. later after 100 gm. glucose orally.. .	1.12	1.10	5.6
Patients on diets low in thiamine chloride			
4. Overnight fasting..	1.63	1.61	2.3
10. " "	0.96	0.93	3.3
Diabetic patients			
12. 14 hrs. after admission in acidosis	2.64	2.65	28.4
13. 52 hrs. after admission in acidosis........	1.03	0.68	18.3
14. 2 hrs. post prandial, uncontrolled	1.67	1.00	7.1
15. Acidosis at admission.	2.20	1.33	32.0

results in Tables II and III also show that there is poor correlation between the concentration of blood acetoacetate and the amount of acetoacetate hydrazone remaining when the overnight waiting period is omitted. By introducing the modifications described into the pyruvic acid determination interference by acetoacetic acid is avoided.

SUMMARY

Elevated concentrations of acetoacetic acid interfere with the determination of blood pyruvic acid. This source of error is overcome by using tungstic acid filtrates and allowing the mixture of hydrazine and hydrazones in ethyl acetate to stand 18 to 24 hours.

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DIFFERENCES IN CALIBRATION CURVES OF ANDROS-
TERONE AND DEHYDROISOANDROSTERONE

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The development by Zimmermann (1) of a colorimetric tech-
nique for measuring the amount of chromogen produced in the
m-dinitrobenzene reaction with those of the sex hormones which
have the active ketone grouping has stimulated investigation of
the crystalline as well as of the urinary androgens. As a conse-
quence of this work, Neustadt (2), Friedgood and Whidden (3),
and Talbot *et al.* (4) have made various adaptations of the Zimmer-
mann technique for use in the Evelyn photoelectric colorimeter.
Spectrophotometric analysis (2) has shown that Filter 520 of the
Evelyn apparatus transmits light at the wave-length corresponding
to the wave-length of the absorption band of the androsterone-*m*-
dinitrobenzene color complex.

In calibration of the Evelyn colorimeter preliminary to measur-
ing the "total sterone" in human urine, both crystalline andros-
terone and dehydroisoandrosterone were studied.[1] We were
especially interested in dehydroisoandrosterone because Lang-
stroth and Talbot (5) reported that it gave the same extinction
coefficient as androsterone. This fact furnished the basis for a
method of determining the respective concentrations of andros-
terone and of dehydroisoandrosterone in human urine. The
value of this observation as applied to clinical work becomes
immediately apparent when it is recalled that Callow (6) reported
that 70 per cent of the total androgens excreted in cases of adreno-

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[1] The androsterone and dehydroisoandrosterone were kindly supplied by
Dr. Erwin Schwenk of the Schering Corporation and Dr. Ernst Oppen-
heimer of the Ciba Pharmaceutical Products, Inc.

genital tumor consisted of dehydroisoandrosterone.   We therefore concerned ourselves with a study which we hoped would help to clarify the differences observed in the reported extinction coefficients of the two substances.

The technique of Neustadt was employed first for measuring the total quantity of "sterone" in urine.   In this method the crystalline hormone was dissolved in 20 cc. of 60 per cent alcohol

TABLE I

Determinations According to Neustadt Method

Androsterone					Dehydroisoandrosterone				
Amount in total volume of colored solution	Galvanometer reading	$L\ (2 - \log G)$	C, mg. per cc. colored solution	K	Amount in total volume of colored solution	Galvanometer reading	$L\ (2 - \log G)$	C, mg. per cc. colored solution	K
mg.					*mg.*				
0.2	92[1]	0.0351	0.007	5.01	0.2	89	0.0505	0.007	7.21
0.3	88[1]	0.0543	0.0107	5.07	0.3	83[1]	0.0796	0.0107	7.43
0.4	85[1]	0.0693	0.0142	4.80	0.4	77[2]	0.1107	0.0142	7.79
0.5	82	0.0862	0.0178	4.84	0.5	72[2]	0.1397	0.0178	7.84
0.6	78[3]	0.1037	0.0215	4.82	0.6	67[2]	0.1707	0.0215	7.89
0.7	75[1]	0.1235	0.025	4.94	0.7	61[1]	0.2129	0.025	8.51
0.8	72[3]	0.1382	0.0285	4.84	0.8	57	0.2441	0.0285	8.56
0.9	68[3]	0.1627	0.0321	5.06	0.9	53[2]	0.2716	0.0321	8.46
1.0	68	0.1675	0.036	4.65	1.0	52[3]	0.2777	0.036	7.71
2.0	47	0.328	0.071	4.62	2.0	29[2]	0.530	0.071	7.46
3.0	33	0.482	0.107	4.56	2.5	22	0.658	0.0893	7.36
4.0	21[2]	0.668	0.142	4.67	3.0	17	0.770	0.107	7.19
6.0	10[2]	0.979	0.2143	4.57	4.0	9[1]	1.034	0.142	7.23
8.0	5[2]	1.260	0.2857	4.41	6.0	3	1.523	0.2143	7.16
Mean................				4.78 ± 0.37					7.70 ± 0.86

and then divided into two 10 cc. aliquots to each of which were added 2 cc. of 2 per cent *m*-dinitrobenzene in 95 per cent alcohol and 2 cc. of 15 per cent aqueous potassium hydroxide.   The tubes were placed in the dark-room and galvanometer readings were taken after 90 minutes.   This time interval was taken as the optimum for the development of the chromogen and these conditions were observed throughout the run.   The calibration curve

for androsterone was found to be similar to that reported by Neustadt. We found that the m-dinitrobenzene reaction with dehydroisoandrosterone was the same as with androsterone as regards the development time and that it gave the same type of reaction curve. However, weight for weight, the chromogen developed with dehydroisoandrosterone was much more intense than that developed with androsterone. This was in agreement with the observation of Friedgood and Whidden (7).

The curves were constructed from the data in Table I which represent the averages of many different experimental runs with various batches of reagents over a period of several months. The color developed by both hormones appears to obey, within certain limits, the law of Beer which is best expressed by the equation $C = L/K$. In this equation C represents the concentration of the hormone per cc. of final volume of colored solution, L represents the negative logarithm of the light transmitted, and K represents the relationship of the chromogen produced, as influenced by specific conditions, to the filter used. We found that the color reaction is not truly linear, because with increasing amounts of either dehydroisoandrosterone or androsterone the chromogen compound developed in the m-dinitrobenzene reaction does not increase proportionately. With androsterone the reaction loses its efficiency at 8.0 mg. With dehydroisoandrosterone efficiency is lost at 6 mg. This would naturally preclude the measurement of material containing concentrations of the respective substances beyond these amounts unless necessary dilutions were made.

The calibration curves (Fig. 1), determined according to Neustadt, show the mg. of androsterone and dehydroisoandrosterone in the total volume of colored solutions plotted on a logarithmic scale against the galvanometer reading which corresponds to the L values in the equation $C = L/K$. The mean K value in a range of 0.2 to 8.0 mg. of androsterone was 4.78 ± 0.37; for dehydroisoandrosterone the mean K was 7.70 ± 0.86.

In order to confirm this observation of a difference in the color development of the two hormones, the work was repeated with the method outlined by Friedgood and Whidden (3). The determinations were carried out in concentrations of 0.045 to 0.3 mg. of androsterone and of dehydroisoandrosterone, which cover the same galvanometer range as in the Neustadt method. The tech-

nique employed in this series of determinations is similar to that of the method of Callow *et al.* (8).   The hormones were dissolved in 95 per cent alcohol and aliquots were chosen so that the amount to be calibrated was contained in 0.2 cc. of 95 per cent alcohol to which was added 0.2 cc. of 2 per cent *m*-dinitrobenzene in absolute alcohol and 0.2 cc. of 2 N aqueous potassium hydroxide.   These
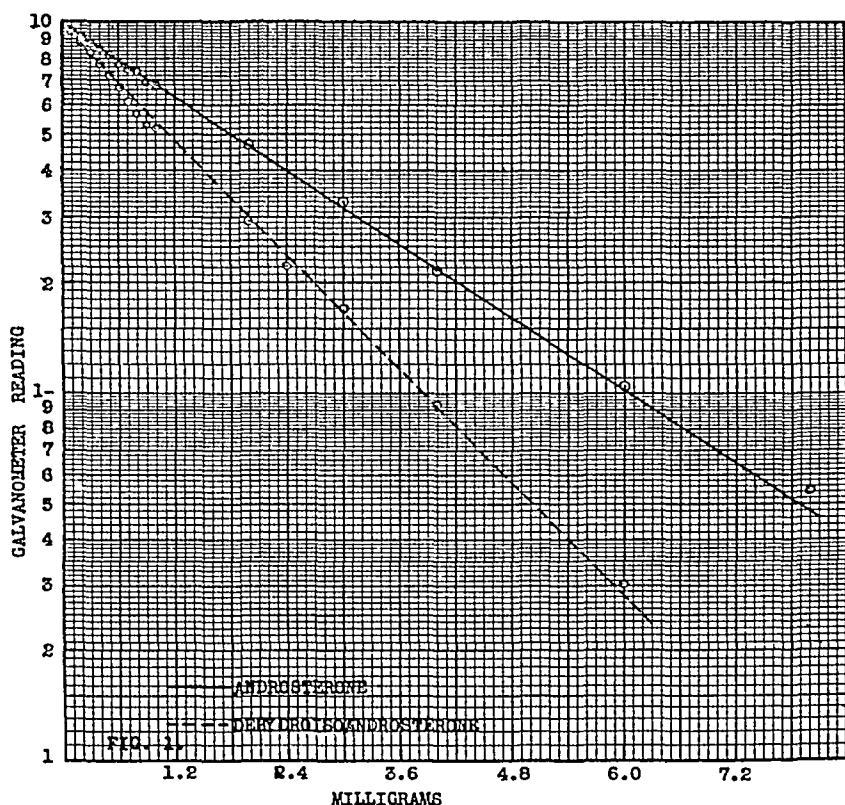
FIG. 1. Curves secured when the method of Neustadt was employed

tubes were kept in a dark-room at 25° and at the end of 75 minutes were diluted with 10 cc. of 95 per cent alcohol, after which galvanometer readings were taken and the calculation for K made. It was again found that weight for weight dehydroisoandrosterone gave a much deeper pink than androsterone, a fact which is in agreement with results of Friedgood and Whidden (7).

Our calibration curve for androsterone was similar to theirs with a mean K for androsterone of 33.29 ± 1.10 and for dehydro-

isoandrosterone 44.35 ± 1.38.   This curve was also plotted on a log scale (Fig. 2) and was based on the data in Table II.

Since the difference between androsterone and dehydroiso-androsterone was apparent with two different techniques, it was decided to check the work spectrophotometrically in order to determine whether this difference was real.   Visual examination
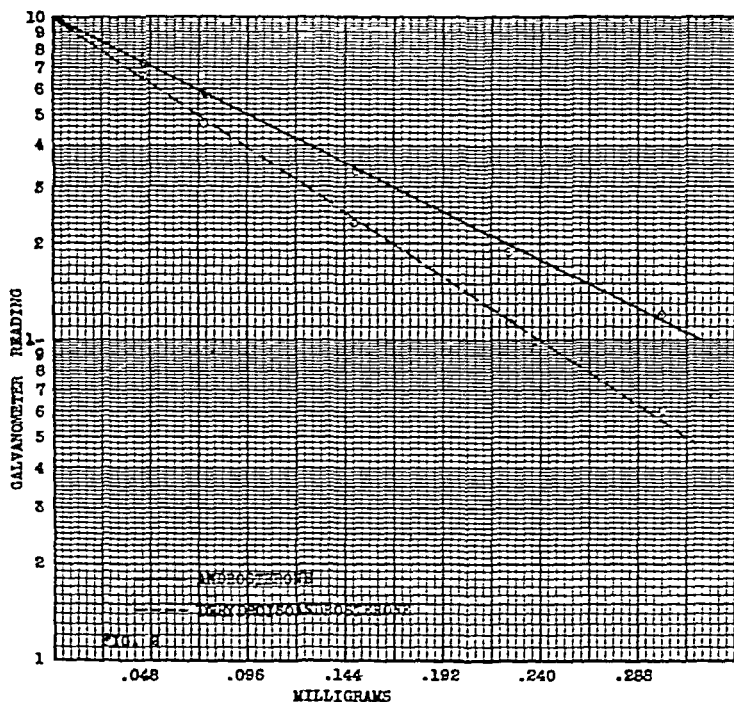
FIG. 2. Curves secured when the method of Friedgood and Whidden was employed.

was carried out with a constant deviation glass spectroscope having a special tungsten lamp source.   It was found that both hormones transmitted light in the 5200 Å. region.   It was observed that dehydroisoandrosterone solutions prepared by both the Neustadt and Friedgood methods showed more absorption in the green portion of the spectrum than did the corresponding androsterone solutions.

As a final check we repeated our studies, employing the method described by Talbot, Butler, and MacLachlan (4).   In this case the two compounds showed calibration curves which could be superimposed and K values which were the same.   Further work by us indicated certain technical factors which we believe may explain the reason for this difference.   Investigations are now in progress and a quantitative spectrophotometric check will be made.

<div align="center">

TABLE II

Determinations According to Friedgood and Whidden Method

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Amount present in total volume of colored solution	Androsterone				Dehydroisoandrosterone			
	Galvanometer reading	L $(2 - \log G)$	C, mg. per cc. colored solution	K	Galvanometer reading	L $(2 - \log G)$	C, mg. per cc. colored solution	K
mg.								
0.045	73[1]	0.1352	0.0042	32.19	66	0.1805	0.0042	42.97
0.075	58	0.2366	0.007	33.80	48	0.319	0.007	45.57
0.150	33[2]	0.475	0.0141	33.62	23	0.638	0.0141	45.26
0.225	18[3]	0.727	0.0212	34.29	11[1]	0.949	0.0212	44.76
0.300	12	0.921	0.0283	32.54	6	1.222	0.0283	43.18
Mean.................				33.29 ± 1.10				44.35 ± 1.38

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Comment

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In an attempt to explain the differences observed by us and those reported by others (5, 2), it was suggested that androsterone and dehydroisoandrosterone be studied in dilutions lower than 1 mg.   We observed, by the Neustadt technique, the same proportionate difference in the entire range from 0.1 to 1.0 mg. that was present in the range of 1 to 8 mg.   With the Friedgood and Whidden method in the range of 0.045 to 0.300 mg. the same proportionate difference in the depth of chromogen produced by the two compounds was observed.   By virtue of our observations that the intensity of the chromogen produced by dehydroisoandrosterone is greater than that for a corresponding amount of androsterone, the practical value of this fact becomes apparent when one attempts to determine specific concentrations of andros-

terone and dehydroisoandrosterone in a given specimen. In the assay of urinary androgens, the calculated amount of "sterone" is based on the K value for androsterone, whereas the intensity of the color reaction may be due to dehydroisoandrosterone as well as other ketosteroids. Therefore, in order to apply the digitonin method described by Langstroth *et al.* (9), we must consider the different K values. This would then require a second computation based on the K value of dehydroisoandrosterone.

SUMMARY

1. There is a difference in the intensity of chromogen produced by androsterone and dehydroisoandrosterone which is apparent by visual spectrophotometry as well as in the calibration curve of the Evelyn photoelectric colorimeter.

2. The K value for androsterone and dehydroisoandrosterone is the same only when the method described by Talbot is used, but is not the same with either the Neustadt or Friedgood method.

3. The difference in K must be taken into consideration in applying the method for determining concentrations of dehydroisoandrosterone in a given urine specimen.

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A COMPARISON OF THE ACTION OF CRYSTALLINE PAPAIN ON NATIVE AND UREA-DENATURED PROTEINS*

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A number of investigators have shown that denatured proteins are digested by enzymes more rapidly than native proteins. Anson and Mirsky (7) have pointed this out specifically for salicylate-denatured hemoglobin and have mentioned it in speaking of denatured proteins in general. The work of Willstätter, Grassmann, and Ambros (31), Lin, Wu, and Chen (23), Cohn and White (15), and others should be noted in this connection. These workers have generally been concerned with egg white or egg albumin, have employed crude enzyme preparations, and have generally studied extensive digestion of the substrate rather than the kinetics of initial digestion. Some of these experiments are difficult to interpret also because they are complicated by the effect of egg white trypsin inhibitor. Deviations in the kinetics of proteolysis that may occur as a result of the presence of this or other inhibitors will be discussed later. In a brief note Linderstrøm-Lang *et al.* (24) have recently reported data indicating that native lactoglobulin may not be attacked at all by enzymes. Since the work reported in this paper was done, Winnick, Davis, and Greenberg (32) have reported data on the digestion of native and urea-denatured ovovitellin, hemoglobin, edestin, ovalbumin, and casein by asclepain. Their studies were primarily concerned with the characterization of the new enzyme rather than the phenomenon of protein denaturation and therefore their experiments were not designed to deal with the particular problems that will be considered in this paper. There has thus been little work

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of a conclusive nature carried out on the digestion of native proteins as compared to denatured proteins (see statement by Anson (2)). Annetts (1), following up the work of Svedberg and Erikson (30), made a careful and valuable analysis of the products formed by the action of papain on native egg albumin, using several physical methods including ultracentrifugation and cataphoresis. This study (which did not include denatured egg albumin) will be mentioned later.

The action of urea and other neutral denaturing agents on proteins has been the subject of considerable research in recent years. Mirsky and Anson (26) prepared urea-denatured protein for various studies. Hopkins (19) studied changes in the chemical reactivity of egg albumin that occur in strong solutions of urea. Burk and Greenberg (13) and Burk (12) have studied the osmotic and other physical relations of several proteins in urea solution (see Greenberg (16)). Steinhardt (29) made a critical study of some phases of the behavior of hemoglobin in urea solutions. Greenstein (17) studied the quantitative "liberation" of —SH groups from proteins in solutions of urea and related compounds, as indicated by the porphyrindin reagent. He reports that the amount "liberated" varies with the protein and ranges from 0 to several equivalents per molecule of protein, and that there is no correlation between the molecular weight changes and the —SH liberated. Anson also has used urea and recently has examined the reducing groups in egg albumin using a detergent denaturing agent, Duponol (4, 5). Papain has been the subject of a similar study in this laboratory (10).

Data in the literature, of the nature referred to above, indicate that certain broad generalizations are justified with regard to the denaturation of proteins by urea or by other means.

1. Physically, changes may occur in the molecular weight, solubility, absorption spectrum, and other properties.

2. Chemically, groups (—SH, —S—S—, and tyrosine phenol) may become "exposed" or become more reactive so that they can be detected by reagents that would show little or no reaction with them when the protein was native (undenatured) (25).

3. Enzymically, proteins that are resistant or only slowly attacked by enzymes when native are readily digested when denatured. Enzymes that have been shown to be proteins lose their catalytic property when they are denatured.

It is the purpose of this communication to extend the third generalization by reporting the apparent relative rates at which papain digests native and denatured Hb,[1] by showing some of the complications of the kinetics of native Hb digestion, by showing that alkali-denatured and urea-denatured Hb are digested at practically the same rate, and by showing that urea in concentrations above that required to denature the protein completely has no further effect on the rate of Hb digestion by papain. Papain is particularly suited to the study of the urea denaturation problem, since Lineweaver and Schwimmer (unpublished data) have found it to be stable in 9 to 10 M urea. Chymotrypsin loses activity rapidly even in 2 M urea (21), while pepsin is stable in 4 M urea (29). The differential effect of denaturing agents on various proteins has recently been discussed and studied by Anson (4, 6).

Methods

Proteolysis was followed by the method of Anson (3). The results are reported in terms of "color value" per 5 ml. of trichloroacetic acid filtrate. The "color value" refers to the change in scale reading on a neutral wedge photometer and is an arbitrary measure of the extent of digestion. The time of digestion was 10 minutes unless otherwise indicated, and the pH was 7.0 ± 0.1. Tyrosine added in the proportion of 0.0008 milliequivalent per 5 ml. of trichloroacetic acid filtrate gives a color value of about 35. This color value is equivalent to about 5 per cent digestion of 2 per cent Hb.

All of the Hb used was prepared according to Anson (3) but was denatured only as indicated. Crystalline papain (9) was used unless otherwise indicated.

DISCUSSION

Comparison of Rate-Substrate Concentration Functions of Native and Denatured Hb and of Relative Rates of Attack on These Two Forms of Hb by Papain—Fig. 1 shows that for practical purposes the maximum rate of digestion of denatured Hb is obtained when the substrate concentration is 2 per cent. This does not appear to be the case for native Hb (inset of Fig. 1). However, the curve in the inset has been constructed from the data of Fig. 2 and is,

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[1] Hb will be used to designate hemoglobin whether native or denatured.

of course, subject to considerable uncertainty because of the variable rate of digestion of native protein (Fig. 2). Nevertheless, calculations based on the enzyme concentrations used in the two cases give a maximum difference in rate of digestion of 2 per cent native and denatured Hb of about 1:6400. If the initial rate, rather than the later constant rate, is used for the comparison, the difference is about 40-fold less (1:100 to 1:200). Since up to
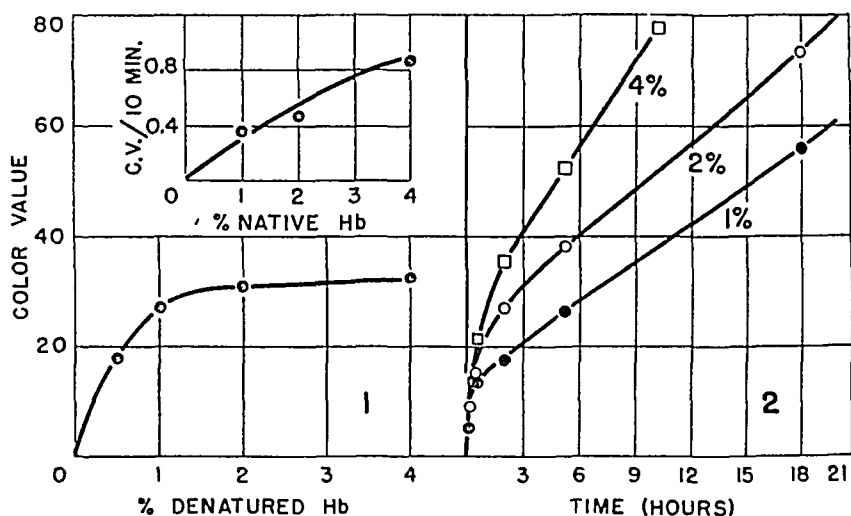
Fig. 1. The relation between the increase in color value and the concentration of urea-denatured Hb. The inset gives the relation for native Hb. (These rates are calculated from the straight portions of the curves in Fig. 2.) The amounts of enzyme N used in the case of denatured and native Hb respectively were 0.002 and 0.2 mg. per 6 ml. of digestion mixture. The specific activity of this enzyme solution which had been prepared for some time appears to be about 30 per cent less than that of the one used in Fig. 5.

Fig. 2. The kinetics of the digestion of 1, 2, and 4 per cent native Hb.

10 per cent of the substrate was digested during the linear portion of the curves in Fig. 2, native Hb rather than an impurity must be disappearing during this time. Winnick, Davis, and Greenberg (32) reported that, for the proteins they used, the denatured forms were digested by asclepain more rapidly than the corresponding native forms. However, the differences do not appear to be as great in general as those observed by the authors for Hb and egg albumin. They were, of course, using a different enzyme and, furthermore, they point out that these particular data are

not suitable for such quantitative comparisons. Kunitz (21) reported that denatured casein is digested about 800 times as fast as native egg albumin by chymotrypsin.

Native Protein Digestion by Papain—Kinetic data similar to those reported in Fig. 2 for Hb have also been obtained for egg albumin. In neither the case of Hb nor that of albumin can the curves obtained by the digestion of the native proteins be superimposed on those obtained by digestion of denatured protein. (Since time and enzyme concentration are interchangeable, Fig. 2 may be compared with Fig. 5.)

Annetts (1) followed the action of papain on native egg albumin by separating the dialyzable constituents from the digestion mixture. She observed an initial rapid rate of digestion, followed by a constant rate, similar to that shown in Fig. 2. Using the alcohol titration method, Willstätter, Grassmann, and Ambros (31) obtained data which yield a similarly shaped curve with time, though their data were not intended to be critical in this region. However, these two quite different methods apparently yield curves similar in shape to those obtained by the authors using trichloroacetic acid-soluble material as a measure of the digestion of native egg albumin by crystalline papain. (Our preliminary curves for egg albumin are similar to those in Fig. 2 for Hb.) Annetts and Willstätter *et al.* carried out the digestions at pH 5.0, while the authors used pH 7.0.

Extrapolation of the linear portions of the curves for the three concentrations of Hb and for egg albumin (unreported) suggests an impurity (perhaps denatured protein) in the substrate. However, the ultracentrifuge data of Annetts (1) indicate that essentially all of the native protein undergoes the loss of a fraction of low molecular weight after which the new fraction of high molecular weight, having an apparent molecular weight about 90 per cent of that of egg albumin, is gradually hydrolyzed.

The following facts further support the contention that native proteins can be directly attacked by enzymes.

(a) Fig. 3 shows that time and enzyme concentration factors are interchangeable in the digestion of native Hb by papain. As pointed out by Bodansky (11), this indicates that either there are no important reactions occurring that are independent of the enzyme, or the rate of the non-enzymic reactions (possibly leading

to equilibrium between two or more forms of Hb) is very high compared with the rate of digestion, so that the experimental kinetics are virtually independent of the non-enzymic reactions. The possibility that the inset in Fig. 1, differing from Fig. 1 proper, represents the digestion of an equilibrium concentration of denatured protein appears to be unlikely on the basis of the observations cited below. The direct interpretation of the inset, of course, is that native Hb has a low affinity for papain.
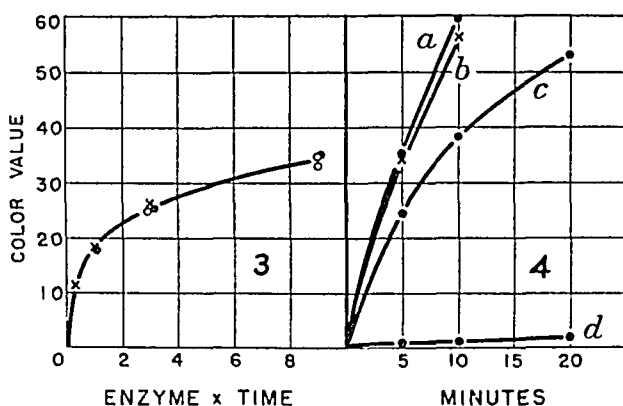
FIG. 3. Comparison of time and enzyme concentration for the action of papain on native Hb. The open circles, solid circles, and crosses correspond to 0.3, 0.1, and 0.033 mg., respectively, of enzyme N per 6 ml. of digestion mixture. The time is in minutes.

FIG. 4. The inhibition of crystalline trypsin digestion of denatured Hb by the antitrypsin of egg albumin. Curve a, control. Curves b and c, 0.14 per cent of egg albumin crystallized twelve times and three times, respectively. Curve d, 0.14 per cent of dried egg white.

(b) The observation of the authors that chymotrypsin attacks native Hb more readily than does papain, whereas papain is about 4 times as active toward denatured Hb as chymotrypsin, leads to the interpretation that some enzymes attack native proteins more rapidly than others. In fact, trypsin is practically inactive towards native egg albumin, whereas chymotrypsin shows definite activity (8, 21). If the digestion represents the hydrolysis of equilibrated denatured protein, then the rates of digestion by papain and chymotrypsin would be expected to stand in the same ratio for native as for denatured protein. At the same time, the rate of digestion of Hb by papain is apparently not limited by the

natural rate of formation of "ordinary" denatured protein, since in that case equal rates of digestion by high concentrations of chymotrypsin and papain would be obtained. The data of Linderstrøm-Lang *et al.* (24) indicate that under certain conditions the rate of digestion of native lactalbumin may be limited by the rate of denaturation. It may also be noted that a specific papain inhibitor that would be destroyed when the Hb is denatured by heat or urea, could, of course, also explain our observation. Refutation of the latter explanation was obtained experimentally by the observation that native 2 per cent Hb only slightly inhibits the digestion of casein by papain. However, this does not mean that the action of inhibitors, and perhaps other factors, can be disregarded as insignificant in all cases. For example, Fig. 4 shows that egg albumin crystallized three times still contains considerable trypsin inhibitor, whereas egg albumin crystallized twelve times is nearly free of inhibitor.

(c) The accumulation of special protein intermediates or products that may result from the action of enzymes on native proteins is illustrated by the conversion of enzyme precursors, trypsinogen (22), pepsinogen (18), and chymotrypsinogen (20) into active enzymes. Kunitz (21) has also studied the autotransformation of active chymotrypsin into active β and γ forms. These results are most reasonably interpreted as due to digestion of native proteins with the production of crystallizable native products resistant to proteolysis. They are also consistent with the idea of Schmidt, Allen, and Tarver (27), who postulate that new proteins are formed by "slight modification rather than *de novo* synthesis from amino acids."

These observations can be explained less simply by assuming the native Hb to be in equilibrium with a denatured Hb that differs enzymically from the various denatured hemoglobins studied in this work, and, furthermore, by assuming the rate of approach to equilibrium to be fast compared to the rate of digestion. However, in the present state of our knowledge essentially the same assumption could be made to support the hypothesis that native proteins will not react with any reagents.

Enzymic Comparison of Urea- and Urea-Alkali-Denatured Hb and Difference between Relative Rates of Digestion of Native and of Denatured Hb by Enzymes—Fig. 5 shows that there is no apparent

difference in the rate of papain digestion of 2 and 4 per cent Hb whether denatured in neutral or alkaline solutions of urea. Since denaturation by urea is a time reaction, the Hb was allowed to stand in 6.6 M urea for at least 60 minutes before use. The color value-enzyme concentration curve may be compared with the data of Anson (3). When a homogenized suspension of heat-denatured Hb was submitted to the action of papain, chymo-
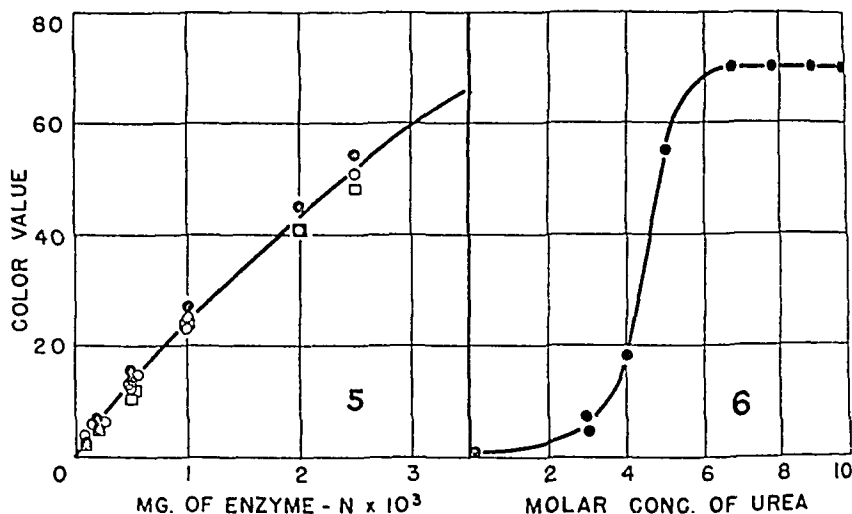
FIG. 5. The relation between the increase in color value and the amount of enzyme N per 6 ml. of digestion mixture. The open circles, solid circles, and squares correspond to 2 per cent urea-denatured, 4 per cent urea-denatured, and 2 per cent alkali-denatured Hb, respectively.

FIG. 6. The relation between the color value and the urea concentration in the digestion mixture. The Hb concentration was 2 per cent and the enzyme N content was 0.0038 mg. per 6 ml. of digestion mixture. The urea-hemoglobin solution was allowed to stand at room temperature for 1 hour before use.

trypsin, and trypsin, the rates of digestion, though somewhat lower for each enzyme, were relatively the same as when urea-denatured Hb was used. The denatured hemoglobins obtained by these different methods of treatment are thus quite similar in digestibility. On the other hand, the factor or factors in native proteins that may be altered by denaturation appear to limit the activity of some enzymes more than others. This is illustrated by the observation, already mentioned, that chymotrypsin and

papain digest native Hb at different *relative* rates than they digest denatured Hb. It is also consistent with the data of Winnick, Davis, and Greenberg (32) from which it appears that the rates of digestion of the five different native proteins by asclepain differ from one another much more than the rates of digestion of the same five denatured proteins.

Rate of Digestion of Hb by Papain As a Function of Concentration of Urea in Substrate Mixture and Increased Lability of Peptide Bonds—Fig. 6 gives the enzyme activity as a function of the concentration of urea in the Hb substrate. The data were obtained by a combination of two experiments. The color values were slightly adjusted so that the two sets of data are superimposed. The increase in amount of water-insoluble protein with urea concentration was measured roughly by dilution at pH 7 after 60 minutes standing and found to be similar to the increase in rate of digestion. However, the various manifestations of change, when proteins are denatured, may not parallel each other quantitatively (Steinhardt (29), Sreenivasaya and Pirie (28), Anson (4)).

From these data the conclusion seems justified that protein —CO·NH— (peptide) bonds become far more available or sensitive to proteolytic enzymes when the proteins are "denatured" (*e.g.*, dissolved in strong urea solution). This conclusion is similar to those reached by Greenstein (17), Anson (5, 6), and Balls and Lineweaver (10) who showed, for various proteins, that —SH, —S—S—, and tyrosine phenol groups may be more reactive in the denatured than in the native protein. On the other hand Chow and Wu (14) have shown for several proteins that there is no change in the number of titratable amino groups on denaturation. These authors used urea as a denaturing agent and the formol titration for the determination of the amino group.

SUMMARY

1. In urea solutions of concentrations greater than 5 to 6 x the initial rate of digestion of Hb by papain is at least 100 times greater than in water solution—the later rate may be as much as 6000 times greater. This increase in digestibility is similar to the decrease in solubility of Hb when it is denatured by urea, but the data obtained are not adequate to prove or disprove an exact parallelism.

2. The following observations make it appear likely that native Hb may be directly attacked by papain, (a) the relative rates of digestion of native Hb by papain and chymotrypsin do not appear to be related to the relative rates of digestion of denatured Hb; (b) the digestion of native Hb is not limited by a rate of denaturation, since the same increase in digestion takes place with either twice the time or twice the enzyme concentration; (c) the formation of enzymes (native proteins) from precursors (native proteins) generally involves proteolysis.

Though denaturation may not· occur before the enzyme acts upon Hb, this does not mean that the enzyme itself does not denature the protein before hydrolysis proceeds.

3. The increase in rate of enzymic hydrolysis of proteins when they are denatured has been found to be comparable to the increase in reactivity of constitutent —SH, —S—S—, and tyrosine phenol groups. The constituent peptide bonds, therefore, may be considered to increase in reactivity in a manner similar to these groups when proteins are denatured.

4. The rate of digestion of denatured Hb by papain is practically the same whether denatured by neutral or alkaline urea or by heat. The increase in the rate of digestion of a protein when it is denatured appears to be different for each enzyme; likewise, the increase in rate for each of several proteins by a single enzyme appears to be different.

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THE CORRELATION OF THE CONFIGURATIONS OF
α-AMINOPHENYLACETIC ACID AND OF ALANINE

By MARTIN KUNA, G. OVAKIMIAN, AND P. A. LEVENE*

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

In 1936 the configuration of tyrosine was correlated to that of aspartic acid in this laboratory and thus the configurations of the natural aliphatic acids were correlated to those of the naturally occurring phenylated amino acids by the methods of classical organic chemistry.[1] The configuration of α-aminophenylacetic acid was at that time correlated to that of the natural amino acids only by an indirect optical method.[2] A method of correlating the configurations of α-amino acids to the corresponding amines was also developed in this laboratory in 1937, and thus the configurations of aliphatic amines were correlated to those of the aromatic amines. Hence it was necessary to convert α-aminophenylacetic acid into the corresponding amine in order that its configuration could be correlated to that of the aliphatic and to the phenylated amino acids. Work in that direction was initiated in 1937, but preliminary to it, it seemed desirable to develop a catalytic method for the reduction of the esters of α-amino acids into the corresponding substituted aminoethanols. While this work was in progress, there appeared a publication of Reihlen, Knöpfle, and Sapper[3] in which the authors correlated the configurations of aliphatic, aromatic, and cyclohexyl derivatives of ethylamines on the basis of optical behavior—a task which, as stated, has been accomplished in this laboratory by methods of classical chemistry. However, the authors anticipated our own experiments in converting

* Died September 6, 1940.

[1] Levene, P. A., and Mardashew, S., J. Biol. Chem., 117, 179 (1937).

[2] Reihlen, H., and Knöpfle, L., Ann. Chem., 523, 199 (1936).

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α-aminophenylacetic acid into the corresponding methylphenyl-aminomethane. The procedure employed by these authors was found in their own eyes to be not very satisfactory and was not recommended by themselves for future work.

$$
\begin{array}{ccc}
\text{COOH} & \text{COOC}_2\text{H}_5 & \text{CH}_2\text{OH} \\
| & | & | \\
\text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \rightarrow & \text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \rightarrow & \text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \rightarrow \\
| & | & | \\
\text{C}_6\text{H}_5 & \text{C}_6\text{H}_5 & \text{C}_6\text{H}_5 \\
\text{Dextro} & \text{Dextro} & \text{Dextro} \\
\text{I} & \text{II} & \text{III}
\end{array}
$$

$$
\begin{array}{ccc}
\text{CH}_2\text{OH} & \text{CH}_2\text{I} & \text{CH}_3 \\
| & | & | \\
\text{CH}_3\text{COOH}\cdot\text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \rightarrow & \text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \rightarrow & \text{HCl}\cdot\text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \\
| & | & | \\
\text{C}_6\text{H}_{11} & \text{C}_6\text{H}_{11} & \text{C}_6\text{H}_{11} \\
\text{Dextro} & & \text{Dextro} \\
\text{IV} & \text{V} & \text{VI} \\
& & \updownarrow
\end{array}
$$

$$
\begin{array}{cccc}
\text{CH}_3 & \text{CH}_3 & \text{CH}_3 & \text{CH}_3 \\
| & | & | & | \\
\text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \rightarrow & \text{HCl}\cdot\text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \rightarrow & \text{HCl}\cdot\text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \rightarrow & \text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \\
| & | & | & | \\
\text{C}_6\text{H}_5 & \text{C}_6\text{H}_5 & \text{C}_6\text{H}_{11} & \text{C}_6\text{H}_{11} \\
\text{Dextro} & \text{Dextro} & \text{Dextro} & \text{Levo} \\
\text{VII} & \text{VIII} & \text{IX} & \text{X} \\
\downarrow & & &
\end{array}
$$

$$
\begin{array}{c}
\text{COOH} \\
| \\
\text{H}_2\text{N}\ldots\text{C}\ldots\text{H} \\
| \\
\text{CH}_3 \\
\text{Dextro}(l) \\
\text{XI}
\end{array}
$$
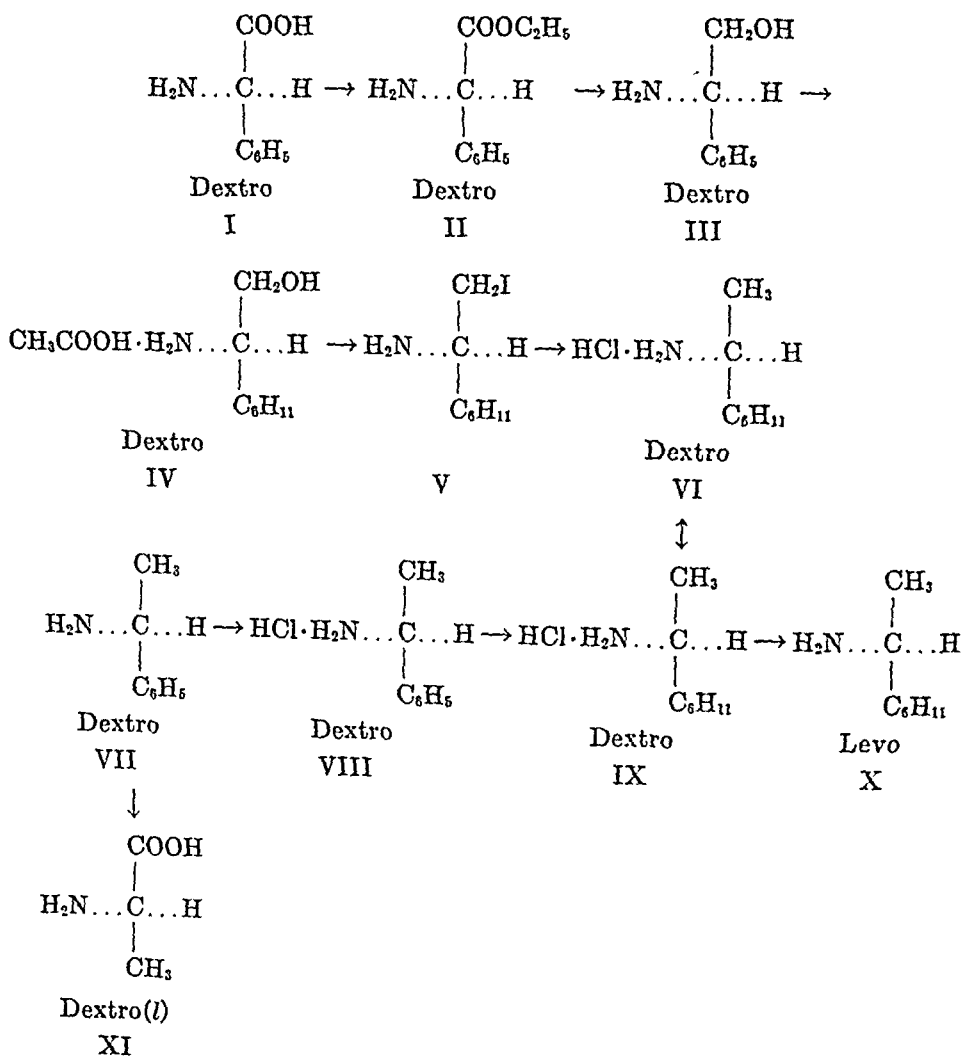
Hence the present study was continued on the plan previously outlined; namely, the 1-phenyl-1-aminoethanol-2 described previously[4] was hydrogenated to the corresponding cyclohexyl deriva-

[4] Ovakimian, G., Kuna, M., and Levene, P. A., J. Biol. Chem., 135, 91 (1940).

tive which in its turn was converted into 1-cyclohexyl-1-amino-2-iodoethane and this without isolation was reduced to 1-cyclohexyl-1-aminoethane. Inasmuch as the correlation of the configurations of the cyclohexyl and the phenylethylamines has been accomplished,[5] and also that of the latter to alanine,[6] it follows that the configuration of α-aminophenylacetic acid is correlated by these means to that of alanine. The results are given in the accompanying set of reactions, showing that dextro-l-α-aminophenylacetic acid is correlated to dextro-l-alanine, in agreement with the results obtained by Reihlen, Knöpfle, and Sapper.[3]

<div align="center">EXPERIMENTAL</div>

Reduction of Aminophenylethanol to Aminocyclohexylethanol Acetate—4 gm. of 1-amino-1-phenylethanol,[4] $[\alpha]_D^{25} = -15.0°$ (in methanol), which remained crystalline at room temperature, were dissolved in 100 ml. of glacial acetic acid (Mallinckrodt A. R.) and 0.6 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 3 days at room temperature. The product was distilled from a micro flask immersed in a bath at a temperature of 110–125°, $p = 1 \times 10^{-4}$ mm. Yield 3.6 gm.

$$[\alpha]_D^{25} = \frac{-0.85° \times 100}{1 \times 11.2} = -7.6° \text{ (in chloroform)}$$

The rotation in methanol was also levo.

$C_{10}H_{21}O_2N$ (203.2).   Calculated.   C 59.11, H 10.46
                             Found.   " 59.24, " 10.44

Reduction of Aminocyclohexylethanol Acetate to Methylcyclohexylaminomethane Hydrochloride—2 gm. of 1-amino-1-cyclohexylethanol acetate, $[\alpha]_D^{25} = -7.6°$ (in chloroform), were dissolved in 35 ml. of a solution containing anhydrous hydrogen iodide (60 gm.) in glacial acetic acid (50 gm.). This was heated in a sealed bomb tube for 4 hours at 125°. Further procedure was as previously described.[7]

[5] Leithe, W., *Ber. chem. Ges.*, **65**, 660 (1932).
[6] Leithe, W., *Ber. chem. Ges.*, **64**, 2827 (1931).
[7] Levene, P. A., and Mardashew, S., *J. Biol. Chem.*, **117**, 707 (1937).

The crystals were dissolved in hot benzene, filtered, and pentane was added to the filtrate.   The crystals weighed 0.9 gm.

$$[\alpha]_{5461}^{25} = \frac{-0.410° \times 100}{1 \times 13.2} = -3.1° \text{ (hydrochloride in water)}$$

$C_8H_{18}NCl$ (163.6).   Calculated.   C 58.69, H 11.09
Found.   " 58.58, " 11.04

N-Acetylaminocyclohexylacetic Ethyl Ester—1 gm. of α-amino-phenylacetic ethyl ester, $[\alpha]_D^{25} = -113°$ (homogeneous), was dissolved in 50 ml. of acetic acid (purified by distillation with acetic anhydride), and 0.5 gm. of Adams' catalyst was added.   This was reduced with hydrogen at 3 atmospheres pressure for 3 days at room temperature.   The product distilled from a bath temperature of 130–140°, $p = 4 \times 10^{-5}$ mm., and crystallized; m.p. 73–75°.

$$[\alpha]_D^{25} = \frac{+0.23° \times 100}{1 \times 5.0} = +4.6° \text{ (in methanol)}$$

$C_{12}H_{21}O_3N$ (227.2).   Calculated.   C 63.38, H 9.32
Found.   " 63.43, " 9.44

The ester group of this substance was not reduced when it was shaken with hydrogen at a pressure of 160 atmospheres, and a temperature of 75° for 9 hours, with Raney's catalyst.   The material was recovered unchanged.

Diacetylaminophenylethanol[8]—2.5 gm. of aminophenylethanol, $[\alpha]_D^{25} = -15.0°$ (in methanol), were dissolved in 40 ml. of methanol, cooled in an ice water bath, and ketene was passed into the solution for 2½ hours.   After evaporation, the semicrystalline material was distilled from a bath temperature of 155–170°, $p = 0.001$ mm. On standing in the cold, the material crystallized.   Yield 2.4 gm.

$$[\alpha]_D^{25} = \frac{-3.47° \times 100}{1 \times 10.8} = -32.1°; \quad [M]_D^{25} = -71.0° \text{ (in methanol)}$$

$C_{12}H_{15}O_3N$ (221.1).   Calculated.   C 65.16, H 6.83
Found.   " 65.27, " 7.26

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[8] Reihlen, Knöpfle, and Sapper[3] report $[M]_D^{20} = +136°$ from an aminophenylethanol hydrochloride of $[M]_D^{20} = +37.9°$.

N-Acetylaminophenylethanol—1.1 gm. of diacetylaminophenylethanol were dissolved in 5 ml. of methanol and 10 ml. of 0.5 N sodium hydroxide. The solution was refluxed for 1 hour and extracted with chloroform. The substance distilled from a bath at a temperature of 170–180°, $p = 4 \times 10^{-4}$ mm.

$$[\alpha]_{5461}^{25} = \frac{-7.28° \times 100}{1 \times 15.6} = -46.7° \text{ (in chloroform)}$$

$C_{10}H_{13}O_2N$ (179.1). Calculated. C 67.00, H 7.32
Found. " 66.85, " 7.44

Diacetylaminocyclohexylethanol—1 gm. of diacetylaminophenylethanol, $[\alpha]_D^{25} = -32.1°$ (in methanol), was dissolved in 25 cc. of glacial acetic acid and 0.5 gm. of Adams' catalyst was added. This was reduced with hydrogen at 3 atmospheres pressure for 3 days at room temperature. The product distilled from a bath temperature of 175–185°, $p = 8 \times 10^{-4}$ mm., $n_D^{25} = 1.4826$.

$$[\alpha]_{5461}^{25} = \frac{+2.35° \times 100}{1 \times 14.1} = +16.7° \text{ (in chloroform)}$$

$C_{12}H_{21}O_4N$ (227.2). Calculated. C 63.38, H 9.32
Found. " 63.22, " 9.41

N-Acetylaminocyclohexylethanol—4 gm. of aminophenylethanol, $[\alpha]_D^{25} = -15.0°$ (in methanol), were dissolved in 100 cc. of acetic acid (purified by distillation with acetic anhydride) and 0.6 gm. of Adams' catalyst was added. This was reduced with hydrogen at 3 atmospheres pressure for 3 days at room temperature. The predominating product was the N-acetylaminocyclohexylethanol, which distilled from a bath temperature of 160–170°, $p = 4 \times 10^{-5}$ mm. Yield 3 gm.; $n_D^{25} = 1.4943$.

$$[\alpha]_D^{25} = 0 \text{ (in methanol)}$$

$$[\alpha]_D^{25} = \frac{+0.71° \times 100}{1 \times 13.6} = +5.2° \text{ (in chloroform)}$$

$C_{10}H_{19}O_2N$ (185.2). Calculated. C 64.74, H 10.34
Found. " 64.98, " 10.69

The forerun contained the acetate and diacetyl derivatives.

The N-acetyl derivative did not react with hydrogen iodide in glacial acetic acid when it was heated for 4 hours at 125°. The product isolated was impure aminocyclohexylethanol hydrochloride. This compound, when re-treated with hydrogen iodide in glacial acetic acid, as above, and with subsequent reduction with Raney's catalyst gave a methylcyclohexylaminomethane hydrochloride of

$$[\alpha]_{5461}^{25} = \frac{-0.130° \times 100}{1 \times 8} = -1.6° \text{ (hydrochloride in water)}$$

GLUCOSE PHOSPHORYLATION AND OXIDATION IN CELL-FREE TISSUE EXTRACTS*

By SIDNEY P. COLOWICK, HERMAN M. KALCKAR,† AND CARL F. CORI

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Dialyzed kidney extracts have been shown to phosphorylate glucose under aerobic conditions when one of a number of oxidizable substrates (such as citrate, glutamate, α-ketoglutarate, succinate), Mg^{++}, adenylic acid, and cozymase are added (1). In particular, the step succinic $- 2H \rightarrow$ fumaric acid was found to bring about phosphorylation. It was then demonstrated that the oxidation of pyruvic acid, which requires catalysis by the succinic-fumaric system, can furnish the energy for the phosphorylation of glucose (2). Since glucose, once it has been phosphorylated, is converted to pyruvic acid in these extracts by the following well known reactions, glucose $+ 2H_3PO_4 \rightarrow$ fructose diphosphate $\rightarrow$ 2 triose phosphate $\rightarrow$ 2 phosphoglyceric acid $\rightarrow$ 2 phosphopyruvic acid $\rightarrow$ 2 pyruvic acid, it was to be expected that, in the presence of a catalytic amount of succinic acid, glucose would be oxidized as readily as pyruvic acid. This was found to be the case in kidney extract (2) and evidence for the same phenomenon has now been obtained in heart, brain, and liver extracts.

Experiments to be reported in the present paper show that the complete oxidation of 1 mole of glucose to CO_2 and H_2O can cause the phosphorylation of 5 moles of glucose to fructose diphosphate. Additional experiments to be reported show that mannose and adenosine can be phosphorylated in kidney extracts.

The methods used were essentially the same as those previously described (1). The tissue was homogenized at 0° with 1.2 to 2.0 volumes of 0.1 M potassium phosphate of pH 7.7 and the resulting paste was centrifuged. The cloudy, cell-free supernatant fluid was in most cases dialyzed for 4 hours against 0.05 M potassium phosphate at 2°. 1 cc. portions of the dialyzed extract were mixed with 0.4 cc. of additions (for details see Fig. 1 and Tables I to VI) and the mixtures were shaken in Warburg
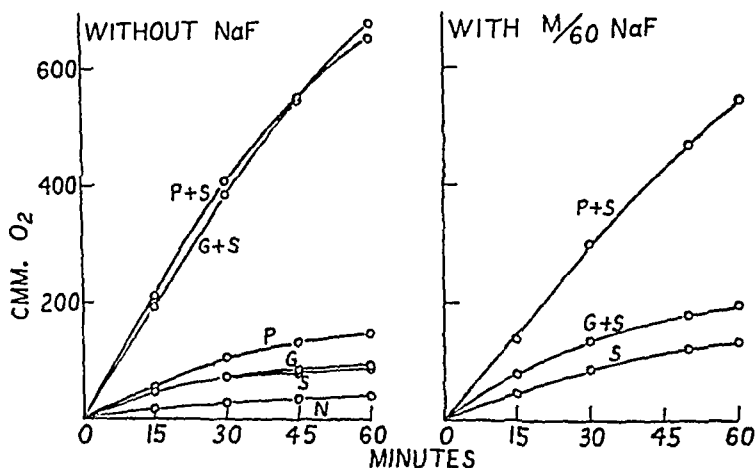
FIG. 1. Oxidation of glucose and pyruvic acid in dialyzed kidney extract. 1 cc. of extract + 2 mg. of $MgSO_4 \cdot 7H_2O$ + 0.3 mg. of adenylic acid + 0.3 mg. of cocarboxylase + other additions as shown. Total volume 1.4 cc.; temperature 37°. N = no additions, S = 0.2 mg. of succinic acid (Na salt), G = 10 mg. of glucose, and P = 7 mg. of pyruvic acid (Li salt).

vessels at 37° in an atmosphere of oxygen. O_2 consumption was calculated for the entire incubation period, a correction being applied to include the oxygen consumed during the equilibration period of 5 minutes. After incubation the samples were analyzed for inorganic phosphate, additional inorganic phosphate liberated by 10 minutes hydrolysis in N H_2SO_4 at 100°, fructose diphosphate (as fructose), phosphoglyceric acid, and free glucose, by the same methods as were used previously. Lactic acid was determined in copper-lime filtrates by the method of Friedemann, Cotonio, and Shaffer as modified by Wendel (3). Pyruvic acid was determined by the method of Clift and Cook (4).

The experiment illustrated in Fig. 1 substantiates and extends the findings of the experiment reported previously (2); it shows that a trace of succinic acid is necessary for the oxidation of glucose and of pyruvic acid[1] in dialyzed kidney extracts and that fluoride prevents the oxidation of the former but not of the latter.   In the case of glucose, succinic acid is necessary (a) for the initial phosphorylation which enables the glucose molecule to be oxidized to phosphoglyceric acid and then transformed to pyruvic

Balance for Glucose Disappearance in Dialyzed Heart Muscle Extract

1 cc. of extract of rabbit heart + 10 micromoles of $MgSO_4$ + 1 micromole of adenylic acid.   Initial inorganic P 48.7 micromoles.   Total volume 1.4 cc.   Incubation time 60 minutes.   The values in the table are expressed in micromoles.

Additions	Glucose disappear-ance	O_2 consumed		P esterified		Total glucose accounted for
			Glucose accounted for		Glucose accounted for	
micromoles						
None		1.5		0		
2 succinate		2.6		0		
57 glucose	15.4	14.3	2.13	18.4	9.2	11.3
57 " + 2 succinate	24.4	22.3	3.47	36.0 (35)	18.0	21.5
45 pyruvate + 2 succinate		31.6				

The figure in parentheses indicates the per cent hydrolysis of the esterified P during 10 minutes in N H_2SO_4 at 100°.

acid and (b) for the oxidation of pyruvic acid which in turn causes further phosphorylation of glucose.   Fluoride prevents the oxidation of glucose by inhibiting the transformation of phosphoglyceric acid to pyruvic acid.   In the absence of NaF, glucose is oxidized as rapidly as pyruvic acid in this extract.

Balance experiments with heart and kidney extracts showed

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[1] The catalysis of pyruvate oxidation by the succinic-fumaric system has been described by Annau and Erdős (5) and by Banga, Ochoa, and Peters (6) and is here confirmed.   It is not definitely known whether the catalysis occurs over the step fumaric → succinic or oxaloacetic → malic acid.

that much more glucose disappeared than was estimated from the oxygen consumption. In an experiment with a dialyzed heart extract (Table I) the respiration without addition of substrate was very low. When glucose was added, a rapid oxidation occurred which was further increased by addition of a catalytic amount of succinic acid.[2] Since 6 micromoles of O_2 are required for the complete oxidation of 1 micromole of glucose, $(14.3 - 1.5)/6 = 2.13$ micromoles of glucose were oxidized without added succinate and $(22.3 - 1.5)/6 = 3.47$ micromoles of glucose were oxidized with addition of succinate, whereas the total glucose disappearance (found by direct estimation) was 15.4 and 24.4 micromoles, respectively. The ratio of glucose disappeared to glucose oxidized was 7.2 and 7.0 in the two cases. In the latter case, 87 per cent of the glucose which disappeared in excess of that oxidized was accounted for as fructose diphosphate. The ratio of moles of fructose diphosphate accumulated to moles of glucose oxidized was 18.0/3.47 or 5.2. Since the energy required for the formation of fructose diphosphate could have come only from the oxidation of glucose, at least ten of the hydrogen transfers involved in the complete oxidation of glucose must have been "coupled"[3] with phosphorylation.

In experiments with dialyzed kidney extracts (Table II) the amount of glucose oxidized by different extracts was quite constant (the values ranging from 3.8 to 4.5 micromoles of glucose per hour). Complete oxidation is indicated by a respiratory quotient of 1.0 for glucose and of 1.25 for pyruvate (calculated 1.20) and by a theoretical oxygen consumption for the oxidation of the latter (2.5 moles of O_2 per mole of pyruvic acid which disappeared).

The glucose which disappeared in excess of that oxidized was found partly as fructose diphosphate, and partly as lactic acid. For example (Experiment 1, Table II) of 16.2 micromoles of glucose which disappeared, 4.5 micromoles were completely oxidized, 2.1 micromoles were found as fructose diphosphate, and 7.3 micro-

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[2] The oxidation of glucose without added succinate in this extract is probably due to traces of succinate (or some other dicarboxylic acid) still present after a relatively short period of dialysis. Longer dialysis could not be employed because it led to inactivation of the heart extracts.

[3] The word "coupled" is used in a descriptive sense without reference to the chemical mechanism involved and it is assumed that each transfer of a pair of hydrogen atoms causes the esterification of 1 atom of P.

moles were converted to lactic acid; 86 per cent of the glucose which disappeared was thus accounted for.

TABLE II

Balances for Glucose and Pyruvic Acid Disappearance in Dialyzed Kidney Extract

1 cc. of extract + 10 micromoles of $MgSO_4$ + 1 micromole of adenylic acid + other additions as indicated. Initial inorganic P content 50 micromoles. Total volume 1.4 cc. Incubation time 60 minutes. The values in the table are expressed in micromoles.

Experiment No.	Additions	Pyruvate disappearance	Glucose disappearance	O₂ consumed	Pyruvate no-counted for	Glucose no-counted for	r.q.	Lactate found	Glucose no-counted for	P esterified	Glucose no-counted for	Total glucose no-counted for
	micromoles											
1	None			2.8								
	26.4 glucose		1.0	6.9				1.8				
	26.4 " + 2 succinate		16.2	28.4		4.5	0.99	14.6	7.3	4.2	2.1	13.9
	22.2 pyruvate + 2 succinate	6.3		18.4	6.2							
2	None			11.5				1.0				
	29.2 glucose + 2 succinate		9.8	36.3		4.1	1.01	5.2	2.1			6.2*
	25.5 pyruvate + 2 succinate	13.2		43.3	12.7		1.25					
3	55.2 glucose		0.3	9.1				1.1				
	55.2 " + 2 succinate		19.2	34.0		4.1		8.0	3.5	11.2	5.6	13.2
	26.9 pyruvate + 2 succinate	13.6		36.8	11.1			2.2				
4	2 succinate			9.5				1.7				
	55 glucose + 2 succinate		24.6	32.0		3.8		23.9	11.1	7.3	3.7	18.6
	54 pyruvate + 2 succinate	18.9		43.5								

* No estimation of esterified P.

The lactic acid formation in these extracts (accounting for from 2.1 to 11.1 micromoles or from 18 to 45 per cent of the glucose which disappeared) is due to a secondary reaction in which part

of the pyruvic acid formed from glucose is reduced to lactic acid by competing with oxygen for the dihydropyridine nucleotide (reduced by the oxidation of triose phosphate). The lactic acid formation is an aerobic phenomenon; an anaerobic conversion of glucose to lactic acid does not occur in kidney extracts.

The ratio, glucose disappeared to glucose oxidized = 6.5, found in one kidney extract (Experiment 4, Table II) is close to

TABLE III

Coupling of Glucose Phosphorylation with Oxidation of Glutamic Acid

1 cc. of dialyzed extract + 25 micromoles of NaF + 10 micromoles of $MgSO_4$ + 1 micromole of adenylic acid + 0.2 micromole of cozymase. Initial inorganic P 90 micromoles for kidney extract, 44 micromoles for heart extract. Total volume 1.4 cc. Incubation time 60 minutes.

Experiment No.	Extract	Additions	O₂ consumed	P esterified	P hydrolyzed in 30 min. in N H₂SO₄
		micromoles	*micromoles*	*micromoles*	*per cent*
1	Rabbit kidney	56 glucose	0.9	0	
		56 " + 35 succinate	15.5	0	
		56 glucose + 70 succinate	19.0	0	
		56 glucose + 27 glutamate	17.0	18.4	
2	Rabbit heart	85 succinate	9.2	0	
		85 " + 56 glucose	14.7	3.6	64
		85 succinate + 34 glutamate + 56 glucose	14.9	18.4	56

the ratio 7.0 found in heart extract (Table I). The lower ratios found in the other kidney extracts may be due to a lack of coupling of some of the oxidative steps with phosphorylation, a possibility which is suggested by the following observation. In Experiment 1, Table III, the oxidation of succinate (15.5 and 19 micromoles of O₂ consumed) caused no phosphorylation, even though fluoride had been added to prevent dephosphorylation, while the oxidation of glutamate (17 micromoles of O₂ consumed) caused a marked phosphorylation. The same phenomenon has

been observed in a dialyzed heart extract (Experiment 2, Table III). In these cases the coupling of succinic acid oxidation with phosphorylation was apparently deficient, while the glutamic acid oxidation was accompanied by phosphorylation. It follows that it must have been the oxidation of glutamic acid either to ketoglutaric or to succinic acid which was linked with phosphorylation.

Experiment 1 in Table IV shows that liver extracts are able to oxidize glucose; phosphate esters did not accumulate in the absence of fluoride. However, a phosphorylation of glucose could be demonstrated by adding fluoride and glutamic acid to dialyzed liver extract (Experiment 2, Table IV).

<div align="center">TABLE IV</div>

<div align="center">*Phosphorylation and Oxidation of Glucose in Liver Extract*</div>

The animals were fasted 48 hours. 1 cc. of liver extract + 1 micromole of adenylic acid. Total volume 1.4 cc. Incubation time 60 minutes. Initial inorganic P 42.5 micromoles.

Experiment No.	Additions	O₂ consumed	P esterified
	micromoles	*micromoles*	*micromoles*
1. Undialyzed extract of rat liver	None	12.4	0
	56 glucose	21.7	0
2. 4 hr. dialyzed extract of rabbit liver	50 NaF	5.2	3.9
	50 " + 56 glucose + 27 glutamic acid	10.4	17.7

A rapid phosphorylation of glucose takes place when pyruvate or succinate is oxidized by a cell-free brain pulp (Table V). In the former case the accumulation of the phosphate ester is as large without as with fluoride, whereas in the case of succinic acid a large accumulation takes place only when fluoride is present; the reason for this difference is at present unknown. In both cases the product of glucose phosphorylation appears, from the values for hydrolyzable P and fructose, to be mainly fructose diphosphate. A small amount of phosphate ester accumulates without addition of glucose when succinate or pyruvate is oxidized in the presence of fluoride. This ester, which has not been identified, is easily hydrolyzable and does not contain fructose.

In a recent note Ochoa (9) reported a coupling of phosphoryla-

tion with pyruvic acid oxidation in brain dispersions with hexose monophosphate as P acceptor.

TABLE V

Glucose Phosphorylation in Brain Pulp

10 gm. of rabbit brain + 12 cc. of 0.1 M potassium phosphate (pH 7.7) homogenized. 1 cc. of pulp + 0.4 cc. of additions. Initial inorganic P 55 micromoles. Incubation time 60 minutes.

Additions	O_2 consumed	P esterified	Esterified P split in 10 min. in N H_2SO_4 at 100°	Fructose calculated from P esterified as fructose diphosphate	Fructose	
					Found	Corrected*
micromoles	*micromoles*	*micromoles*	*per cent*	*micromoles*	*micromoles*	*micromoles*
Fixed at once					0	0
56 glucose	16.0	7.4	22	3.7	2.3	5.2
68 succinate	41.2	0		0	0	0
91 pyruvate	36.1	0		0	0	0
56 glucose + 68 succinate	43.6	14.2	18	7.1	3.4	7.7
56 glucose + 91 pyruvate	35.2	49.5	23	24.7	9.3	21.1
48 NaF	11.5	2.3		1.1	0	0
48 " + 56 glucose	12.4	17.4	22	8.7	3.2	7.3
48 NaF + 68 succinate	31.2	6.8	50	3.4	0	0
48 NaF + 91 pyruvate	17.7	5.2	75	2.6	0	0
48 NaF + 68 succinate + 56 glucose	33.8	52.2	25	26.1	8.3	18.9
48 NaF + 91 pyruvate + 56 glucose	20.3	50.5	25	25.2	8.4	19.1

* Fructose diphosphate gives only 44 per cent of the color given by an equivalent amount of free fructose in the Seliwanoff reaction as modified by Roe (7). The readings were made with a Summerson (8) photoelectric colorimeter, with Filter 54.

Two new substrates, mannose and adenosine, have been added to the list of compounds (10) which can be phosphorylated in kidney extract. In Table VI it is shown that, in the presence of

NaF, oxidation of succinate causes a very large phosphorylation of both glucose and mannose. The product in both cases is fructose diphosphate, as indicated by the ratio of esterified P to fructose as well as by the hydrolysis values. Since mannose-1-phosphate, synthetically prepared, is not converted to mannose-6-phosphate by tissue extracts (11), mannose is probably phosphorylated primarily in position 6. Jephcott and Robison (12) showed that when mannose is fermented by dried yeast large amounts of mannose-6-phosphate accumulate. The mannose-6-phosphate is then changed by the enzyme "isomerase" (Lohmann (13)) to

TABLE VI

Phosphorylation of Mannose and Adenosine in Kidney Extract

1 cc. of dialyzed kidney extract + 1 micromole of adenylic acid + 10 micromoles of $MgSO_4$ + 50 micromoles of NaF + 85 micromoles of sodium succinate. Initial inorganic P 50.0 micromoles. Total volume 1.4 cc. Incubation time 60 minutes.

Additions	P esterified	Esterified P hydrolyzable in 10 min. at $100°$ in N H_2SO_4	Fructose	
			Found	Corrected*
micromoles	*micromoles*	*per cent*	*micromoles*	*micromoles*
H_2O	1.9		0	
56 glucose	47.4	30	9.7	22.0
56 mannose	49.7	28	10.2	22.7
39 adenosine	13.2	34		

* See foot-note, Table V.

the equilibrium ester of glucose-6- $\rightleftarrows$ fructose-6-phosphate, and the latter undergoes phosphorylation to fructose diphosphate.

The product of phosphorylation of adenosine is hydrolyzed 34 per cent in 10 minutes, indicating that it is a mixture of adenosine monophosphate (0 per cent hydrolyzable) and adenosine polyphosphates (50 to 67 per cent hydrolyzable). Ostern *et al.* (14) described the phosphorylation of adenosine in yeast; it has not been observed previously in animal tissues.

The aerobic phosphorylation of glucose observed in tissue extracts probably takes place over the adenylic acid system. As demonstrated by Kalckar (10) adenylic acid can take up inorganic phosphate in kidney extract under aerobic conditions, forming adenosine triphosphate. The transfer of the labile phos-

phate groups of the latter to glucose could not be demonstrated in kidney extract (1, 10), but has now been found to take place in heart extract. Of 0.29 mg. of acid-labile P added as adenosine triphosphate to 1.4 cc. of a dialyzed pig heart extract in the presence of glucose and 0.0002 M iodoacetate, 0.10 mg. was found as inorganic P and 0.10 mg. as acid-stable P after anaerobic incubation for 45 minutes at 37°. In the absence of glucose, 0.22 mg. of inorganic P and no acid-stable P were formed.

DISCUSSION

In the present paper is described a system in which the energy for the phosphorylation of glucose is furnished by the oxidation of part of the phosphorylated sugar to CO_2 and H_2O; while 1 molecule of glucose is completely burned in a cell-free and dialyzed heart extract, 5 molecules of fructose diphosphate accumulate.[4] It is therefore of interest to examine the individual steps in the oxidation of glucose which might be expected to cause phosphorylation. The oxidation of glucose is dependent on its phosphorylation which leads to the formation of fructose diphosphate $\rightleftarrows$ 2 triose phosphate. The first oxidation is that of triose phosphate to glyceric acid phosphate, the formation of which has been demonstrated previously in extracts poisoned with fluoride (1). The second oxidative step involves pyruvic acid and requires a catalytic amount of C_4 dicarboxylic acid. Fluoride, which prevents the transformation of phosphoglyceric to pyruvic acid, inhibits the oxidation of glucose in tissue extracts.

The main path of pyruvate oxidation in animal tissues is not definitely known; it may involve a condensation with another organic acid to a di- or tricarboxylic acid; e.g., pyruvate + oxaloacetate − 2H → citrate + CO_2. The citrate cycle proposed by Krebs (15), which represents one of the possible paths, involves the following oxidative steps; (a) isocitrate − 2H → α-ketoglutarate + CO_2, (b) α-ketoglutarate − 2H → succinate +

[4] This corresponds to a coupling of ten of the hydrogen transfers involved in the oxidation of glucose with phosphorylation. The ratio of glucose disappeared to glucose oxidized is 7 in these experiments. This suggests that twelve steps are linked with phosphorylation and that some hexose diphosphate disappears again in side reactions (e.g., conversion to lactic acid).

CO_2, (c) succinate $- 2H \rightarrow$ fumarate, (d) malate $- 2H \rightarrow$ oxalo-acetate.

The oxidation of citrate and α-ketoglutarate has been shown to cause phosphorylation of glucose (1, 16) but the isolated steps (a) and (b) have not yet been studied. (c) has definitely been shown to cause phosphorylation of glucose (1) and (d) has been shown to give rise to phosphopyruvic acid (10). Analogous results have been obtained by Belitzer and Tsibakova (17), who described the phosphorylation of creatine in minced and washed rabbit heart coupled with the oxidation of citrate, α-ketoglutarate, succinate, and pyruvate. By means of arsenite, which prevents other oxidations, they were able to show that the step succinic $\rightarrow$ fumaric acid may be linked with phosphocreatine synthesis.

The reaction by which inorganic phosphate is taken up is known in the case of glyceraldehyde phosphate oxidation; Negelein and Brömel (18) and Warburg and Christian (19) showed that the carbonyl group reacts with inorganic phosphate and that its oxidation (by diphosphopyridine nucleotide) leads to the formation of diphosphoglyceric acid. The phosphate attached to the carboxylic group is then transferred to glucose over the adenylic acid system, the over-all result being the oxidation of 1 molecule of triose phosphate coupled with the esterification of 1 molecule of inorganic phosphate. Lipmann (20) has recently shown that the carbonyl group of pyruvic acid reacts with inorganic phosphate and then undergoes oxidation, yielding acetyl phosphate which serves as phosphate donor over the adenylic acid system. The mechanism of the phosphorylation coupled with the first two steps of glucose oxidation is thus fairly well understood. Whether a similar mechanism exists at later stages of glucose oxidation has not been investigated.[5]

The complete oxidation of 1 molecule of glucose involves the removal of twelve pairs of hydrogen atoms. Since 10 moles of P can be esterified during this process, it might be concluded that the efficiency of the phosphorylation observed here is practically maximal. However, as Belitzer and Tsibakova (17) have pointed out, the possibility remains that not only the pri-

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[5] The oxidation of α-ketoglutarate to succinate, which is analogous to the oxidation of pyruvate to acetate, would yield succinyl phosphate as the primary oxidation product.

mary removal of hydrogen from the substrate but also the further steps in the transfer of hydrogen from pyridine, thiamine, etc., to oxygen, may be linked with phosphorylation. If this were the case, the ten phosphorylating steps observed here would be only a fraction of the total number of phosphorylations which might be linked with glucose oxidation. Belitzer and Tsibakova, in support of the latter view, have found that the uptake of 1 atom of O can be associated with the esterification of from 2 to 3.5 atoms of P in minced heart muscle. Ochoa (21) has shown that in brain extracts, when pyruvate is oxidized, 2 atoms of P can be esterified per atom of O consumed, whereas when succinate is oxidized the ratio is 1:1. Similarly, in heart extract, we have found ratios higher than 1 with pyruvate as substrate, and ratios of 1 or lower with succinate as substrate.

The "aerobic glycolysis" which is observed in kidney extracts might be due to several factors. The reaction between triose phosphate and pyridine nucleotide may occur faster than the reaction of dihydropyridine with flavoprotein → cytochrome system → oxygen; this would permit pyruvate to act as H acceptor, a reaction which is dependent on the activity of the lactic dehydrogenase. The oxidation of pyruvate may be slow, so that it accumulates in sufficient amounts to compete with oxygen as H acceptor. Which of these possibilities is realized has not been investigated. The basic reaction here, as in heart extract, is the esterification of 5 to 6 molecules of glucose for each molecule of glucose burned; the lactic acid formation is a secondary reaction.

In the simplified system represented by tissue extracts the energy derived from oxidations, if it is used at all, can be used only for phosphorylations, since other reactions which require energy do not occur. In the normal cell, under aerobic conditions, hexose diphosphate does not accumulate and lactic acid is not formed; this means that the energy derived from oxidations instead of being used exclusively for the transfer of phosphate to glucose is diverted to a variety of other cellular functions.[6] This is merely an expression of the regulatory function of the cell which permits it to direct the flow of energy into various channels. The

[6] Glucose, in order to be converted to glycogen, must first be phosphorylated; in this case a considerable part of the oxidative energy may be used for glucose phosphorylation.

aerobic glycolysis which occurs in "damaged" cells may be due to a derangement of this regulatory function which results in the utilization of a greater percentage of oxidative energy for glucose phosphorylation.

SUMMARY

1. Extracts of heart muscle and kidney oxidize glucose to carbon dioxide and water. For 1 mole of glucose which is completely burned, an additional 6 moles of glucose disappear, 5 of which are identified as fructose diphosphate in experiments with heart extract. This indicates that at least ten of the hydrogen transfers involved in the complete oxidation of glucose are linked with phosphorylation. In kidney extracts, which exhibit no anaerobic glycolysis, aerobic glycolysis occurs; i.e., a large part of the hexose diphosphate formed from glucose is changed to lactic acid. From 70 to 90 per cent of the glucose which disappears is accounted for in these experiments with heart and kidney extract.

2. The oxidation of glucose in these extracts is preceded by phosphorylation and follows the path triose phosphate $\rightarrow$ phosphoglycerate $\rightarrow$ pyruvate $\rightarrow$ CO_2 + H_2O. The oxidation of glucose is prevented by fluoride, which inhibits the transformation of phosphoglycerate to pyruvate. The oxidation of pyruvate requires catalysis by the succinic-fumaric system and since glucose is oxidized over pyruvate it also requires a catalytic amount of fumaric acid for its oxidation.

3. Liver extracts are able to phosphorylate and to oxidize glucose. In the presence of fluoride and glucose, hexose diphosphate accumulates when glutamic acid is used as the oxidizable substrate. In brain extracts a phosphorylation of glucose to fructose diphosphate, linked with the oxidation of pyruvate or succinate, has been demonstrated.

4. The oxidation of succinate leads to the phosphorylation of mannose and adenosine in kidney extracts; the former is converted to fructose diphosphate, the latter to a mixture of adenylic acid and adenosine polyphosphate.

5. An anaerobic transfer of the labile phosphate groups of adenosine triphosphate to glucose in the presence of monoiodoacetate has been demonstrated in heart extract.

6. The relation of these findings to the utilization of oxidative energy in the cell is discussed.

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SOME ADDITIONAL OBSERVATIONS ON THE SPECIFICITY OF CHOLINESTERASE

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As an extension of previous investigations (1, 2) concerning the specificity of cholinesterase, the present study deals with the effect upon enzymatic hydrolysis of variations in the chain length of the acid component of choline esters.

It is known from earlier work (3, 1) that increasing the normal chain length from 2 to 4 carbon atoms results in increased enzymatic and decreased alkaline splitting. However, nothing has been reported concerning the hydrolysis of the successively higher members of the series. It was shown (1) that the dl-caproyl-β-methylcholine chloride is hydrolyzed, enzymatically, over 4 times as fast as the corresponding acetyl ester, while the latter undergoes nonenzymatic scission at a rate over 4 times that of the former. In the same communication it was reported that isovaleryl-β-dimethyl-γ-homocholine iodide was hydrolyzed over 3 times as fast as the corresponding acetyl derivative, while the non-enzymatic splitting of the latter was about 1.5 times that of the former.

In this paper the preparation and hydrolysis of the esters of the normal 5-, 6-, and 7-carbon acids will be described as well as the succinic and maleic acid derivatives. Of these, to the author's knowledge, only valerylcholine has been prepared previously. The β-bromoethyl esters serving as intermediates for the preparation of the choline esters have not been described before, and these as well as the choline ester chloroplatinates have also been prepared.

EXPERIMENTAL

The manometric method with the Warburg apparatus and horse serum as the source of enzyme was employed for the hydrolysis studies as already described (1).

β-Bromoethyl Esters—These were prepared by refluxing an excess of the acid anhydride with ethylene bromohydrin on a steam bath. The oily product was washed in a separatory funnel with cold water, treated with cold Na_2CO_3 solution followed by cold $CaCl_2$ solution, and then dried with anhydrous Na_2SO_4. Final purification was effected by vacuum distillation (Table I). The esters were all colorless oils with the exception of the maleate which was a colorless solid. The latter compound was purified by four crystallizations from absolute alcohol.

Choline Bromide Esters—The appropriate β-bromoethyl ester was added to an excess of anhydrous $(CH_3)_3N$ in dry benzene. The cooled reactants were placed in a brown glass tube which was kept in a salt-ice bath; the tube was sealed, and allowed to stand

TABLE I

Preparation of β-Bromoethyl Esters

β-Bromoethyl esters	Fraction collected by vacuum distillation		Percentage Br	
	Temperature	Pressure	Theory	Found
	°C.	mm. Hg		
Valerate.....................	112–114	25	38.2	38.4
Caproate.....................	124–126	23	35.8	35.9
Heptoate.....................	138–140	24	33.7	33.5
Succinate.....................	216–217	26	48.1	48.0
Maleate.....................	66 (m.p.)		48.4	48.4

at room temperature for 2 days protected from direct light. Crystals of the choline ester salt which formed were washed with dry ether, dissolved in absolute alcohol, and precipitated by addition of dry ether. This process was repeated two times; the final product was quickly filtered off by suction, and dried in a vacuum desiccator over P_2O_5. The esters of the monobasic acids are deliquescent, but those of the dibasic acids are not.

In some cases addition of ether to the alcoholic solution of the choline ester yielded an oil instead of the solid. It was found that if acetone were substituted for the alcohol, ether precipitation gave solids. Hence the final precipitation was made from acetone solution in the cases of caproate, heptoate, and succinate.

It may be seen from the analytical data in Table II that the

Br content of the heptoate is above the calculated value, while the caproate and valerate contain Br in more nearly the theoretical percentage. The impurity responsible is relatively rich in Br, is less soluble than the desired compound, and can be removed to a great extent by discarding the first material precipitated by the ether. However, it could not be completely eliminated even after repeated fractional precipitations. Fortunately, the impurity had little effect upon the hydrolysis studies, under the conditions employed, since fractions containing a higher percentage of the Br were split at approximately the same rates as the purer compounds. For example, a fraction containing 30 per cent Br was split by the enzyme at a rate corresponding to the liberation of 118 c.mm. of CO_2 compared to 125 c.mm. for the fraction containing 28.3 per cent.

TABLE II

Preparation of Choline Bromide Esters and Their Chloroplatinates

Choline bromide ester	Percentage Br		M.p. of Pt salt	Percentage Pt	
	Theory	Found		Theory	Found
			°C.		
Valerate................	29.8	30.6	211	24.9	24.7
Caproate.... ..	28.3	29.0	204–206	24.0	24.7
Heptoate......	27.0	28.3	198–200	23.2	23.3
Succinate........	35.5	35.5	222 (decomposed)	28.0	27.3
Maleate....	35.7	35.0	230 "	28.0	28.4

The choline ester bromides, rather than the chlorides, were prepared because of the greater tendency of the latter to form oils difficult to purify.

Choline Ester Chloroplatinates—These salts were prepared from $PtCl_4$ and the choline ester bromides. As indicated in the preceding paper (2), an excess of $PtCl_4$ results in displacement of the halogen from choline ester bromides or iodides, yielding the compounds, $PtCl_6[N(CH_3)_3CH_2CH_2OCOR]_2$ and $PtCl_6[(N-(CH_3)_3CH_2CH_2OCO)_2R']$, corresponding to esters of mono- and dicarboxylic acids respectively. Each chloroplatinate was precipitated by mixture of the absolute alcohol solutions of the two reactants. The precipitate was washed repeatedly with absolute alcohol and finally with dry ether, and the product was dried *in vacuo* over P_2O_5.

DISCUSSION

The effect upon both enzymatic and alkaline hydrolysis of increasing the number of carbon atoms in the n-acyl group is apparent from Fig. 1. It might be pointed out that in an earlier communication only slight enzymatic scission was observed with the 16-carbon acyl ester when a suspension of this rather insoluble compound was tested (1).

The values for the hydrolysis of the succinyl- and maleylcholine esters, compared to acetylcholine, are given in Table III. A possible explanation of the weaker enzyme action in these cases
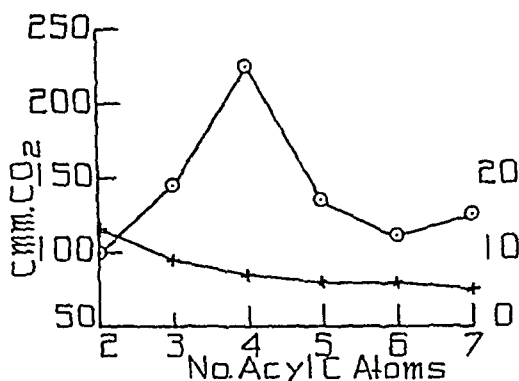
Fig. 1. Enzymatic and non-enzymatic hydrolysis of n-acyl choline esters. ⊙, enzymatic scission corresponding to the left-hand scale of ordinates. ✕, non-enzymatic scission corresponding to the right-hand scale of ordinates. The ordinates are expressed as c.mm. of CO_2 liberated in 30 minutes at 30° in a total volume of 4 ml. containing 1 per cent substrate and 0.5 per cent horse serum. Data for the esters of the 2-, 3-, and 4-carbon acids were taken from an earlier paper (1).

is that the proximity of the two acyl groups in the molecule constitutes a steric configuration that makes it difficult for active centers on the colloidal enzyme particles to come into contact with the ester linkages they are to affect. Apparently the unsaturated ester is split faster.

One might expect that those choline esters more rapidly hydrolyzed by cholinesterase would exhibit weaker or at least more transient biological effects on tissues containing this enzyme. That this is not necessarily the case, however, was demonstrated with the acetyl esters of thiocholine and two of its derivatives (2). Another example is to be found in the data given in this

paper when compared with the biological effects of acetyl-, propionyl-, butyryl-, and valerylcholine reported by Chang and Gaddum (4). These workers found that the effect on blood pressure of the rabbit decreased as the molecular weight of the ester increased, and that the same was true for the effect upon contraction of the isolated rabbit intestine. Were there a correlation between enzymatic scission and physiological response, the butyryl compound would have produced less effect than the valeryl ester. Furthermore, enzyme action appears to play no rôle in the activity of the esters on frog rectus muscle, since Chang and Gaddum found little difference in effect with or without the presence of eserine, and eserine practically abolishes cholinesterase activity. Additional lack of correlation is seen in the fact that propionylcholine elicited the strongest response from frog rectus muscle.

TABLE III

Effect of Additional Structural Changes on Hydrolysis of Choline Esters

Ester	Hydrolysis (30 min., 30°, 4 ml. total volume, 1 per cent substrate), c.mm. CO_2	
	Non-enzymatic	Enzymatic (0.5 per cent serum)
Acetylcholine chloride....	13	100
Succinylcholine bromide...	27	4
Maleylcholine bromide	120	13

In the case of leech muscle, propionylcholine was less potent in producing contractions than either acetyl- or butyrylcholine.

SUMMARY

The β-bromoethyl and choline bromide esters of valeric, caproic, heptoic, succinic, and maleic acids were prepared, as well as the chloroplatinates of the choline esters.

From previous data plus those presented in this paper, it follows that enzymatic scission of *n*-acyl choline esters increases with lengthening of the hydrocarbon chain to the butyryl compound, and decreases thereafter. The esters of the dicarboxylic acids are split relatively slowly; the succinyl compound was hydrolyzed at a slower rate than maleylcholine.

In regard to the *n*-acyl choline esters from acetyl to valeryl inclusive, there appears to be no relation between the enzymatic hydrolysis found in this work and the biological effects reported by Chang and Gaddum (4).

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THE RIBOFLAVIN CONTENT OF BLOOD AND URINE*

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Although a variety of methods for determining riboflavin has
been proposed and numerous assays on a wide range of biological
material carried out, very few data are available regarding the
riboflavin content of the blood and urine of various animal species,
and especially of human subjects. This is probably attributable
in part at least to the difficulty of estimating accurately the small
amounts of riboflavin in these materials.

The present paper reports a study of, first, the applicability
of the bacterial method of Snell and Strong (1) to blood and urine,
second, some observations on the amount of riboflavin found in
the blood and urine of human adults and of several animal species,
and third, the effect of varied intake on the urinary excretion of
riboflavin by the human.

EXPERIMENTAL

The assays were carried out by the bacterial method as pre-
viously described (1). The samples were diluted to a convenient
volume, and suitable aliquots pipetted directly into the culture
tubes. For bloods this dilution was usually 5- to 10-fold. The
samples were preserved during the interval between collection and
analysis by adding small amounts of toluene and chloroform (or
thymol in a few cases) and placing them in a cold, dark place.
Control experiments indicated that in the quantities used these

preservatives had no influence on the assay. Alkalinity in the urine samples was avoided by addition of 3.0 cc. of glacial acetic acid to each 24 hour sample as it was collected. The necessity of this precaution was demonstrated by the loss of approximately 50 per cent of the riboflavin from a urine of pH 8.5 to 9.0 during 3 days storage in the refrigerator.

Bloods—When whole, hemolyzed blood was assayed as above, the incubated assay tubes contained a large amount of red-brown, fibrous solid matter, which made titration difficult, and apparently altered the normal growth of the bacteria, since low results were

· TABLE I

Microbiological Determination of Riboflavin in Blood and Urine

Material and preparation	Diluted solution added	Titration, 0.1 N alkali ·	Riboflavin	
			Per tube	Per gm. sample
	cc. per tube	cc. per tube	γ	γ
Oxalated dog blood;	1.0	4.1	0.112	1.12
diluted 10 times and	1.0	4.0	0.109	1.09
allowed to hemolyze	1.5	5.5	0.165	1.10
	1.5	5.3	0.158	1.05
	2.0	6.4	0.200	1.00
	2.0	6.2	0.192	0.95
Human urine; diluted	1.0	1.8	0.050	0.50
10 times with water	1.0	1.9	0.053	0.53
	2.0	3.0	0.093	0.47
	2.0	2.7	0.082	0.41
	4.0	5.7	0.190	0.48
	4.0	5.3	0.175	0.44

obtained on tubes containing over 0.2 cc. of blood. However, concordant and reproducible values were obtained by using less than this amount of blood per tube, and titrating in the presence of a large amount of indicator. For this purpose a brom-thymol blue solution of 5 times the usual strength was used. Alternatively the precipitate was filtered off, washed, and the filtrate titrated. The agreement at different levels is indicated by the data in Table I.

The results of such assays on a variety of blood samples are recorded in Table II. Each figure is the average of values from duplicate tubes at three different levels. Assays on ten samples

of blood obtained over a 6 week period from an additional human subject showed 0.42, 0.51, 0.48, 0.53, 0.40, 0.45, 0.43, 0.48, 0.44, and 0.54 γ, with an average of 0.47 γ per cc. respectively.

Evidence for the validity of the results in Table II was obtained by recovery experiments on whole blood. On ten samples of human blood recoveries of added riboflavin as determined by direct assay were 126, 108, 103, 124, 106, 106, 106, 98, 116, and 88 per cent respectively. Each figure is the average result from six

TABLE II

Riboflavin Content of Bloods

All figures are in micrograms per cc. of whole blood.

Sample No.	Hog	Calf	Rat	Dog	Human
1	1.03, 0.87, 0.93, 0.91, 1.00; (0.95)*	0.46, 0.60, 0.63, 0.56, 0.52, 0.55, 0.52; (0.55)*	0.41	1.08, 0.83; (0.96)*	0.51, 0.51, 0.54; (0.52)*
2		0.43	0.43	0.91	0.50, 0.54; (0.52)*
3		0.39	0.51	1.00	0.43
4		0.41		1.08, 0.92; (1.00)*	0.49
Average	0.95	0.44	0.45	0.97	0.49

* These figures were obtained by repeated assays on samples of whole oxalated blood which were kept in a refrigerator and preserved by the addition of approximately 0.1 cc. of chloroform per 100 cc. of blood. Some of the above determinations were made as long as 2 months after the sample had been collected. The figures in parentheses are averages.

assay tubes. Similar experiments on animal bloods gave the following per cent recoveries: calf blood 121, 128, 132, 114, 118, 101; hog blood 121, 128; rat blood 99, 116; dog blood 105 and 136. It is apparent that the recoveries, especially on the animal bloods, were somewhat too high.

Attempts to separate the riboflavin from the bulk of the blood solids by an extraction procedure were at first unsuccessful. Autoclaving with as high as 50 volumes of water, or with various concentrations of acid, and centrifuging off the insoluble material after

neutralization yielded extracts containing variable amounts of riboflavin, but never more than about half of the total amount present in the original blood as determined by direct assay. Repeated washing of the insoluble matter or attempts to elute with 0.1 N HCl-methanol (1:1) gave only traces of additional riboflavin. Furthermore, when pure riboflavin was added to whole blood and attempts made to extract it as described above, low and variable recoveries ranging from 50 to 93 per cent resulted.

A preparation showing substantially the amount of riboflavin indicated by direct assay was, however, obtained from a sample of blood by the following procedure. A mixture of 15 cc. of human blood, 100 cc. of water, and 1 cc. of 10 per cent HCl was centrifuged 1 hour, and the supernatant liquid decanted from the small gummy precipitate. The supernatant was stirred $\frac{1}{2}$ hour with 2 gm. of Lloyd's reagent, centrifuged, and the adsorbent combined with the above precipitate. The filtrate, which still contained the bulk of the blood solids, contained no riboflavin, and when known amounts of riboflavin were added to it, quantitative recoveries were obtained. The combined adsorbent and precipitate were found to contain riboflavin equivalent to 0.64 γ per cc. of the original blood. Direct assay of the blood gave 0.54 γ per cc.

Urines—Preliminary experiments to establish the validity of the bacterial determination of riboflavin in urine are summarized in Table III. The photolysis was accomplished by exposing a 1 cm. layer of basified urine (pH 9.0) for 48 hours at a temperature of 25° or less to light from a 100 watt bulb at a distance of 25 cm. This treatment destroyed over 95 per cent of the riboflavin originally present. Photolysis at neutrality in the presence of chloroform as an antiseptic was attempted, but only 75 per cent of the riboflavin was destroyed in 72 hours. The adsorption on Lloyd's reagent was carried out by stirring 100 cc. of acidified urine (pH 2.0) for 1 hour with 2 gm. of the adsorbent and filtering. Over 98 per cent of the riboflavin was thereby removed. In this connection it was found that riboflavin adsorbed on Lloyd's reagent could be very successfully assayed by adding the adsorbate directly to the assay tubes.

It will be noted that riboflavin added to whole urine was quantitatively recovered, and furthermore that known amounts were accurately determined in the presence of urine from which the riboflavin had been removed by photolysis or adsorption on

Lloyd's reagent. No difficulty was experienced in obtaining concordant results at different levels (Table I). It therefore seems very probable that the bacterial assay provides reliable values for the riboflavin content of urine.

In order to ascertain the urinary excretion of riboflavin by normal adults, assays were made on a number of 24 hour samples

TABLE III

Determination of Riboflavin in Urine

Sample No.	Urine analyzed		Highest amount of urine per assay tube	Riboflavin		Recovery
	Species	Preliminary treatment		Added	Found	
			cc.	γ per cc.	γ per cc.	per cent
1	Human, 24 hr. samples	None	0.20		1.02	
		"	0.10	1.00	2.00	98
2		"	0.50		0.34	
					0.31	
		"	0.30	0.25	0.59	106
		"	0.30	0.33	0.67	104
		Photolyzed	1.00		0.01	
		"	0.50	0.20	0.20	100
		Adsorbed on Lloyd's reagent	4.00		0.002	
		" "	2.50	0.033	0.032	96
3		None	0.20		0.71	
		"	0.05	1.0	1.71	100
4	Guinea pig	"	0.10		2.10	
		"	0.05	2.0	4.28	107
		Photolyzed	2.00		0.12	
		"	1.00	0.10	0.22	100
5		None	0.10		1.50	
		"	0.05	2.00	3.36	93
6	Dog	"	0.10		1.50	
7		"	0.10		2.50	

obtained from University students and faculty. The results are collected in Table IV.

A study was also made of the urinary riboflavin excretion of four young college women on a diet supplying known amounts of the vitamin. This diet was planned for the primary purpose of studying nitrogen balance in human subjects.[1] Each member of the

[1] This experiment was under the direction of Professor M. S. Reynolds of the Department of Home Economics.

"diet squad" consumed a constant, weighed quantity of the food, so that the daily protein intake was 25 gm. The results are summarized in Table V. Urinary excretions on an unrestricted diet can be seen in Table IV. The experimental diet was eaten by each subject for 3 days before any urine samples were collected. During most of the experimental period the urines were assayed only every 2 days, consecutive 24 hour samples being pooled. It should be noted, however, that the results in Table V are expressed as micrograms ingested or excreted per 24 hours. On the 16th day 2 mg. of pure riboflavin were given orally, and on the 17th

TABLE IV

24 Hour Urinary Excretion of Riboflavin by Human Adults on Unrestricted Diets

Subject No.	Sex	Excretion	Subject No.	Sex	Excretion	Subject No.	Sex	Excretion
		γ			γ			γ
1	Male	750	7	Female	576	11	Female	477
2	Female	530	8	Male	480	11	"	630
3	"	667	9	Female	680	11	"	2248†
4	"	684	10	"	550	12	"	602
5	"	480	11	"	587	12	"	697
6	"	547	11*	"	704	12	"	780
						12	"	833

* Samples collected on different days from the same subject are indicated by repetition of the subject number. The subjects were selected from University students and faculty.

† Approximately ¼ pound of beef liver was ingested by Subject 11 on this day.

day, 5 mg., and during this period the urine samples were analyzed daily. The intakes were in each case determined by bacterial assays on representative portions of the diet.

Blood riboflavin determinations were carried out on Subjects 3, 4, and 5 on the 15th day, and the values 0.33, 0.35, and 0.29 γ per gm. respectively were found.

The response of several other normal human subjects and of several clinical patients to oral administration of pure riboflavin is shown in Table VI. Normal excretions of Subjects 1, 2, and 11 have already been given in Table IV. Of the clinical patients,

TABLE V

24 Hour Urinary Excretion of Riboflavin on Controlled Diets

Blood riboflavin determinations were carried out on Subjects 3, 4, and 5 on the 15th day. and the values 0.33, 0.35, and 0.29 γ per gm. respectively were found.

Days	Subject 3		Subject 4		Subject 5		Subject 6	
	Intake	Excretion	Intake	Excretion	Intake	Excretion	Intake	Excretion
	γ	γ	γ	γ	γ	γ	γ	γ
1, 2	1653	560	1653	320	1464	320	1464	380
3, 4	1653	460	1653	300	1464	360	1464	320
5, 6	1653	290	1653	150	1464	240	1464	270
7, 8	1130	146	1258	94	1258	76	1125	196
9, 10	1130	102	1258	98	1258	78	1125	132
11, 12	1130	74	1258	56	1258	54	1125	76
13, 14	1927	118	1938	92	1869	104		
15	1927	120	1938	154	1869			
16*	3927	560	3938	440	3869	380		
17†	6927	3780	6938	3280	*			
18	1927	560	1938	470				

* 2 mg. of riboflavin were administered orally at 9.00 a.m.

† 5 mg. of riboflavin were administered orally at 6.00 p.m.

TABLE VI

24 Hour Urinary Excretion of Pure Riboflavin Administered to Human Subjects

Subject No.	1st day		2nd day		3rd day		4th day	
	Administered*	Excreted	Administered*	Excreted	Administered*	Excreted	Administered*	Excreted
	γ	γ	γ	γ	γ	γ	γ	γ
1	5000	4250	5000	3190	None	1510	None	1110
2	5000	4720	5000	4500	"	1090	"	870
11	None	587	None	704				
11	"	700	"	770	5000	3300	5000	1940
13	"	19	"	21				
13	"	60	"	50	5000	1180	5000	2640
14	"	832	6000	1760	6000	4650	6000	5520
15	3000	220	3000	†	3000	2324	3000	798
16	4000	364	4000	106	4000	98		

* In addition to dietary intake.

† Sample lost.

Subject 14, age 29, was suffering from a skin disease diagnosed as pemphigus; Subject 15, age 47, was an achondroplastic dwarf with a history of malnutrition; and Subject 16, age 18, was in the 8th month of pregnancy and was suffering from congenital syphilis, nausea, vomiting, and malnutrition. The urinary riboflavin output of Subject 14 was followed for approximately 3 weeks prior to the initiation of riboflavin therapy, and found to average 250 γ per 24 hours (extremes 98 to 399 γ). One 24 hour sample from Subject 15 obtained 2 days before the riboflavin therapy was started contained 57 γ of riboflavin. Subject 13 was an apparently normal young woman whose urinary riboflavin excretion on her usual diet was abnormally low (see Table VI, 1st and 2nd days).

DISCUSSION

Since assays on blood in which over 0.2 cc. of the blood was added per tube usually gave lower results than the values obtained on quantities less than 0.2 cc., some doubt existed as to which figure should be trusted. It was for this reason that attempts were made to separate the riboflavin from the bulk of the blood solids so as to obtain preparations easy to assay. The attempt was successful in one case, and the riboflavin content of the preparation was found to be close to that determined directly on the whole blood. The failure of the simpler extraction procedures is understandable in view of the high protein and low riboflavin content of blood. The probability that more riboflavin was actually present than could readily be extracted was strengthened by the observation that added riboflavin was also not quantitatively extracted by similar procedures. It is believed that the above evidence, the successful recovery of added riboflavin (at least in human bloods), and the good agreement at different levels below 0.2 cc. per tube indicate that the direct bacterial assay of blood furnished a reliable measure of the riboflavin present.

All the human bloods assayed contained in the neighborhood of 0.5 γ of riboflavin per gm., except in the case of the three subjects on restricted riboflavin intake. Rat and calf bloods contained about as much of the vitamin as the human blood, while the amount found in dog and hog bloods was about twice as high. Very few values are available in the literature to compare with these results. Ochoa and Rossiter (2) found 50 to 65 γ of allox-

azine-adenine dinucleotide per 100 cc. of rat blood, a figure equivalent to 0.20 to 0.27 γ of riboflavin per cc. Fraser *et al.* (3), using the bacterial method of assay, found the blood of rats and dogs on normal diets contained 0.22 and 0.63 γ of riboflavin per cc. respectively. However, they regarded the results as of questionable value because of substances in the bloods which inhibited growth of the bacteria.

The daily urinary excretion of riboflavin by normal human adults appears to be of the order of 500 to 800 γ on unrestricted diets. This value rapidly decreased to 50 to 150 γ on a dietary intake of 1 to 2 mg. of riboflavin per day in the case of four normal young women. These data would appear to indicate that such an intake of riboflavin is at best no more than marginal, and perhaps insufficient satisfactorily to supply the daily requirement. When extra amounts of riboflavin (2 to 5 mg. per day) were given orally to normal persons, the bulk of it was promptly excreted (Table V), but in the case of several individuals whose urinary excretion suggested a low level of riboflavin nutrition this response was delayed or absent (Tables V and VI).

Previous studies of urinary riboflavin excretion have yielded similar results. Helmer (4) reported excretions of 120 and 175 Bourquin-Sherman units in the pooled 24 hour urine samples of two persons. If 1 Bourquin-Sherman unit is regarded as equal to 3 γ of riboflavin, these figures are equivalent to 360 and 525 γ respectively. The assays of Roscoe (5) probably measured other B vitamins as well as riboflavin. Emmerie has reported values ranging from 276 to 1250 γ in several normal human urines (6). Fraser *et al.* (3) and Vivanco (7) found sharp decreases in the urinary excretion of dogs and rats on lowered intakes, and increases followed the administration of riboflavin. Emmerie (6, 8) has made similar observations on several human subjects.

SUMMARY

1. The applicability of the bacterial riboflavin assay method to blood and urine has been established.

2. The content of riboflavin in the blood and urine of normal individuals of human and other species has been determined.

3. The response of urinary excretion of riboflavin to increased and decreased intakes has been studied.

The authors wish to extend grateful acknowledgment to Professor M. S. Reynolds for the opportunity to study the urinary riboflavin excretion in the metabolic study directed by her and to the members of the diet squad, Miss Elizabeth Peterson, Miss Josephine Gardner, Miss Catherine Walliker, and Miss Marion Sorrenson.

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THE CARBOHYDRATE COMPONENT OF
THE RICE FACTOR

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In a previous publication (1) evidence was presented to show that the growth-stimulating effect of polished rice or cartilage could be duplicated by two substances, glycine and chondroitin, fed jointly. Each of these compounds was found necessary for the optimal growth of the chick. Further evidence (2) was obtained which showed that the active constituent of chondroitin is glucuronic acid. We have also found that a number of other substances are able to serve as the carbohydrate moiety of the "rice factor." These include certain vegetable gums, pentoses, and hexuronic and hexonic acids.

Methods and Results

The basal diets varied somewhat as the work progressed simultaneously in both laboratories. These modified diets, which differed in minor respects in composition of the salt mixture, kind of vegetable oil, and nature of the principal energy source, were found to yield similar results when compared. The following diet is representative: water-washed fish meal 15.0, water-washed casein 10.0, gelatin 5.0, salts 4.5, brewers' yeast 10.0, sardine oil, fortified, 0.5, soy bean oil 5.0, cerelose (glucose) or starch and sucrose to 100.0, hexane extract of dried alfalfa equivalent to 1 per cent.

White Leghorn chicks were used as the experimental animals. They were housed in electrically heated, wire-floored, metal battery brooders. The experimental test period varied from 2 to

4 weeks.   When the shorter periods were used, they were preceded by a 1 or 2 week depletion period during which chicks were fed only the basal diet.   Eight to twelve chicks were used in each group.   Glucuronic acid was prepared by the method of Quick (3).   The other supplements were commercial products which were in some cases tested by polariscope.   The growth of chicks receiving each of the supplements was expressed in per cent of the growth made by chicks fed only the basal diet.   This permits a comparison of data obtained in different series of tests.   The results are summarized in Table I.

TABLE I

Activity of Various Supplements As Sources of Carbohydrate Component of Rice Factor

Supplement	Level	No. of trials	Basal gain	Supplement	Level	No. of trials	Basal gain
	per cent		per cent		per cent		per cent
None................		15	100	d-Arabinose.........	0.5	4	121
Gum arabic..........	5	4	128	l-Arabinose.........	0.5	3	111
" "	10	5	135	"	1	5	125
Sodium alginate......	2	4	118	d-Ribose............	0.5	1	105
Gum tragacanth......	5	3	80	Rhamnose...........	0.5	1	100
Pectin...........	2	2	95	Glucuronic acid*....	0.5	1	129
d-Xylose.............	1	8	113	Calcium gluconate...	2	3	120
"	3	6	114	" " ...	4	1	129
				Galactonic lactone...	1	2	130

* Other data on glucuronic acid have been previously reported (2).

After glucuronic acid had been found effective in promoting growth, vegetable gums known to be rich in uronic acids were tested.   Gum arabic which contains 14 per cent glucuronic acid (4) and sodium alginate which yields on hydrolysis approximately 90 per cent mannuronic acid (5) were found effective.   As gum arabic also contains large amounts of l-arabinose, and since uronic acids can be decarboxylated to pentoses, it seemed possible that the latter might also promote growth.   It was subsequently found that d-xylose, d-arabinose, and l-arabinose all caused a growth response.   Calcium gluconate and galactonic acid lactone were similarly effective.

On the other hand, gum tragacanth and pectin, which contain 33 and 67 per cent of combined galacturonic acid, respectively (6), gave no increases in growth. Probably the growth-promoting effect of any one of these gums will depend on its digestibility in the chick, as well as on composition.

Generally, the supplements most effective at the lower levels have been d-glucuronic acid and d-arabinose. Glucuronic and mannuronic acids can be regarded as interchangeable on the basis of a common enolic form. The conversion of either of these into arabinose would be a complicated process, although possible. There is a simpler theoretical relation between gluconic and galactonic acids and arabinose. By decarboxylation of these two acids, 5-carbon alcohols are produced which on oxidation of a terminal group can yield arabinose

SUMMARY

A number of substances were found capable of serving as the carbohydrate component of the "rice factor" for chicks. These include gum arabic, sodium alginate, glucuronic and gluconic acids, galactonic lactone, arabinose, and xylose.

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A STUDY OF CHOLESTEROL ESTERASE IN LIVER AND BRAIN*

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In 1916 Bloor (1) called attention to the possibility that cholesterol may take an active part in fat metabolism, and in recent years he has inclined to the view that fatty acids are transported in part as cholesterol esters (2). This hypothesis has recently been vigorously supported by Schramm and Wolff (3) on the basis of evidence concerning cholesterol esterases: the presence of esterifying factors in serum (4) and in pancreas (5, 6), and the discovery of a cholesterol ester-hydrolyzing factor, active at pH 5.3, in liver and other tissues by Klein (7). Schramm and Wolff concluded from this evidence that cholesterol-esterifying systems are present in extracellular spaces (blood stream, intestinal lumen) and hydrolyzing systems within the cells, and they advanced the hypothesis that fatty acids are esterified with cholesterol in the intestinal lumen before absorption into the cells of the mucosa[1] and that fatty acids are carried from the blood into

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* Some of the experiments were carried out in the Chemical Laboratory of the Babies Hospital, with the aid of a grant from the Carnegie Corporation of New York.

[1] Because of his finding that a large part of ingested free cholesterol could be recovered in the thoracic chyle as cholesterol esters (8) and that free cholesterol was esterified on incubation with pancreas in the presence of fat (5), Mueller suggested that the esterification of free cholesterol with fatty acids in the lumen of the intestine might be of fundamental importance in absorption of cholesterol. Brockett, Spiers, and Himwich (9) concluded from their finding that a marked rise in cholesterol in the lymph accompanies the increase in fatty acids following fat feeding that cholesterol aids in the absorption of fatty acids, probably by forming esters with the fatty acids in the intestinal lumen.

tissue cells as cholesterol esters. It was implied that this is the major, if not the sole, mechanism for the transport of fatty acids into cells. If proved to be tenable, this hypothesis is manifestly of fundamental importance in lipid biochemistry.

There can be no doubt of the existence of a cholesterol-esterifying factor in blood serum. We have found it in all species studied and the finding has been verified in several other laboratories. Further evidence for the presence of an esterifying factor in the pancreas has been supplied in the present study with the Sym (10) technique in which non-aqueous solvents are employed.

The experiments described in this communication are of interest chiefly in their bearing on the third piece of evidence on which the hypothesis of Schramm and Wolff was based; i.e., the presence of a cholesterol ester-hydrolyzing factor in tissue cells. They were undertaken originally because of the following consideration: if the esterification of cholesterol takes place continuously in the blood serum in vivo as it does in vitro (4), there must be a continuous flow of cholesterol esters from the serum into the tissues, and, since cholesterol esters do not normally accumulate in the tissues, the latter must contain active cholesterol ester-hydrolyzing systems. Most of the work was carried out on two tissues, the liver because of its well known active lipid metabolism, and the brain because of its unique position in containing the largest average concentration of cholesterol of any organ and no cholesterol esters.

The experiments with liver yielded two positive results. (a) On incubation of emulsions or suspensions of liver an esterification of cholesterol occurred. (b) In mixtures of the same preparations with blood serum there was hydrolysis of cholesterol esters. Both reactions took place in the same pH range on the acid side of neutrality. The latter result represents a qualitative confirmation of Klein's findings (7); but we were unable to demonstrate the high activity of liver preparations in hydrolyzing the cholesterol esters of blood serum reported by him, and our results indicate that the enzyme has an optimal activity at a considerably lower pH than he stated.

Several investigators (11–14) have reported the presence of a cholesterol ester-hydrolyzing factor in liver, but Mueller (15) and Wendt (16) were unable to find it. Our results together with

those of Klein (7) show beyond doubt that such a factor occurs in liver and thus appear to support the hypothesis of Schramm and Wolff. However, the finding that a cholesterol-esterifying system is also present in liver complicates the picture and thus tends to weaken the argument which they have advanced.

Our inability to obtain any evidence for the presence of cholesterol esterase in brain does not favor the hypothesis of Schramm and Wolff. It is possible of course that the enzyme is more labile in this organ than in liver and that we have not hit upon the proper conditions for its action, or, most likely, it is of very low activity, as might be anticipated from the very slow rate of turnover of cholesterol in adult brain (17). Furthermore, if the brain synthesizes its own fatty acids (17), cholesterol esterase would not be needed for the mechanism proposed by Schramm and Wolff.

One of the outstanding facts of cholesterol biochemistry—the constancy of the proportion between combined and free cholesterol in blood serum (18)—is difficult to correlate with this hypothesis. For, if fatty acids are carried largely as cholesterol esters, one might expect changes in the ratio during periods of intensive fatty acid metabolism, as during the absorption of fat. Schramm and Wolff (3) explained the constancy of the proportion by assuming that the cholesterol, set free from cholesterol esters coming into the cells from the serum, is returned to the serum for re-esterification. This mechanism may provide an adequate explanation under physiological conditions, but in diseases such as hypothyroidism and nephrosis in which changes of several fold in the total cholesterol concentration of the serum may take place within a few days, it might be expected that this equilibrium would break down. However, in the absence of secondary factors such as infection, no significant variation in the ratio of combined to free cholesterol occurs. Some mechanism besides that proposed by Schramm and Wolff must be involved in maintaining the equilibrium during such episodes.

Except for the hydrolysis of cholesterol esters in dog serum in the presence of bile salts (19) reactions catalyzed by cholesterol esterases proceed at a very slow rate. If cholesterol took such an active part in the transport of fatty acids as is suggested by Schramm and Wolff, one might expect to find more active cholesterol esterases in animal tissues.

The general procedure consisted in incubating at 37–38° various tissue preparations (emulsions, extracts, slices, etc.) either alone or with substrates, usually blood serum. Total and free cholesterol concentrations were determined before and after incubation (20). At least two (frequently three or four) replicate determinations were carried out in most experiments. As many of the preparations were difficult to pipette, total cholesterol determinations were subject to considerable error. All calculations of percentage esterification or hydrolysis were based, therefore, on the ratio of combined to free cholesterol which is independent of errors in measuring the sample. pH was determined with the glass electrode apparatus of Rosebury (21) or the Beckman instrument which were checked frequently against standard buffers. Sodium ethyl mercurithiosalicylate was used as a preservative throughout the investigation.

Experiments with Tissue Emulsions. Liver—Rat livers were emulsified in approximately 4 volumes of water with the apparatus of Potter and Elvehjem (22) and incubated. The pH decreased from a starting level of 6.4 to 6.9 to values which varied considerably from rat to rat. The early results showed considerable esterification of free cholesterol and suggested that the degree of esterification increased as the pH decreased. Attempts to control the pH by making emulsions in buffers were unsuccessful; no esterification occurred, perhaps because such emulsions were unstable. The pH was varied to some extent by fasting some of the rats before they were killed and by adding different amounts of glucose to one of the emulsions (Rat 8). The results (Table I), which are tabulated in order of increasing pH, show that a significant esterification of cholesterol usually occurred and that there was some tendency for the degree of esterification to be greater in the emulsions with the lower pH values. In the hope of increasing the esterification nine emulsions were agitated during incubation by rotation in test-tubes on a wheel. Significant esterification occurred (6.9 to 36.7 per cent) but a comparison of rotated and non-rotated portions of the same emulsion showed a considerable inhibition of the esterifying factor by agitation.

To rule out the possibility that the esterifying factor of blood serum was responsible for the finding emulsions were prepared

from liver which had been perfused with saline until the perfusate was colorless and from liver slices which had been washed eight times with Krebs' solution. Esterification occurred as in untreated liver preparations. Furthermore, we have confirmed Klein's (7) finding that no esterification occurs in serum buffered to pH 5.3.

Twenty rat liver emulsions were mixed with equal parts of blood serum (rat or human) and incubated. No esterification occurred and in all but two of the mixtures some hydrolysis of cholesterol esters took place (maximum 32.7 per cent, average 11.4 ± 8.0 (s.d.) per cent). The large variation bore no apparent relation to the final pH which ranged from 4.05 to 6.63. Two

TABLE I

Esterification of Cholesterol in Liver Emulsions

Rat No.	Time of incubation	pH at end of incubation	Esterification	Rat No.	Time of incubation	pH at end of incubation	Esterification
	days		*per cent*		*days*		*per cent*
1	3	3.95	19.3	8	3	4.92	6.2
9	3	4.40	32.5	8	3	5.00	3.9
10	3	4.49	27.9	8	3	5.04	8.1
8	3	4.58	15.1	3	2	5.20	10.2
8	3	4.59	9.5	8	3	5.42	2.6
2	4	4.61	27.9	4	2	5.58	8.9
8	3	4.79	1.3	6	3	5.88	5.0
11	3	4.83	27.2				

samples of liver were emulsified directly in blood serum and incubated. No esterification or hydrolysis occurred. (The final pH was 5.38 and 5.30.) Other experiments with liver emulsions will be described in the section on tissue extracts below.

Brain—Emulsions of brain were prepared as described for liver, except that a somewhat larger proportion of water or buffer solution (5 to 10 volumes) was used, and incubated. In seven emulsions of mouse brain in water and twenty-two emulsions of rat brain with and without buffers no esterification of cholesterol occurred. The pH ranged from 4.33 to 7.68.

No hydrolysis of cholesterol esters could be demonstrated in a series of experiments in which rat brain emulsions were incubated with blood serum.

Experiments with Tissue Extracts. Saline Extracts of Liver—We have repeated the experiments of Klein (7) on the livers of several animal species and have confirmed his findings qualitatively, except in the rat (Table II). However, hydrolysis of cholesterol esters of the magnitude reported by him (70 to 100 per cent with beef liver extracts) was not approached. A possible reason for the discrepancy was our use of centrifugation in accord with our interpretation of Klein's statement that the organ *extracts* were prepared "in the usual way" without further specification. Such centrifuged extracts contain much less cholesterol than apparently was supplied to the mixtures by Klein's preparations,[2] and it seems likely, therefore, that Klein employed tissue *suspensions*, decanted

TABLE II

Hydrolysis of Cholesterol Esters by Saline Extracts of Liver in Mixtures with Blood Serum

Animal	No. of animals	pH*	Hydrolysis of cholesterol esters		
			Minimum	Maximum	Average*
			per cent	per cent	per cent
Beef........	7	5.38 ± 0.07	6.4	22.9	14.5 ± 5.6
Dog........	6	5.30 ± 0.08	2.5	10.8	6.5 ± 3.3
Rat........	11	5.41 ± 0.21	0.0	4.7	2.2 ± 1.8
Pig.........	8	5.27 ± 0.03	7.5	24.7	14.2 ± 5.4
Sheep......	6	5.38 ± 0.05	8.1	10.3	8.8 ± 0.9

* The standard deviation was calculated from the equation $\sigma = \sqrt{\Sigma d^2/(n-1)}$.

from the sand without further treatment. This interpretation was borne out by experiments with such suspensions prepared from five rat livers. In contrast with the negative results with centrifuged extracts there was an average hydrolysis of 19.4 ± 5.4 (s.D.) per cent (minimum 13.4, maximum 24.8).

Since Klein obtained the greatest hydrolysis with beef liver, we repeated our experiments with this species, using both uncentrifuged suspensions and centrifuged extracts from the same preparations. In addition emulsions in distilled water were prepared as described in the preceding section from the same

[2] It is difficult to interpret Klein's data, as he refers to the total cholesterol in the mixtures as serum cholesterol.

samples of liver and substituted for the suspensions in identical experiments. The emulsions and uncentrifuged saline suspensions were also incubated alone for 3 days. (The mixtures with serum and buffer were incubated 1 day (7).) One cat liver was

TABLE III

Effect of Liver Suspensions, Extracts, and Emulsions on Synthesis and Hydrolysis of Cholesterol Esters

Mixture	Beef Liver 1		Beef Liver 2		Beef Liver 3		Beef Liver 4	
	pH	Hydroly-sis*	pH	Hy-droly-sis*	pH	Hy-droly-sis*	pH	Hy-droly-sis*
		per cent		*per cent*		*per cent*		*per cent*
Suspension + serum + buffer.....	5.41	23.5†	5.38	33.1	5.41	37.1	5.42	24.3
Suspension + serum + acid buffer‡ .	4.71	44.0					4.68	35.4
Centrifuged suspension + serum + buffer	5.43	32.0	5.41	27.3	5.31	24.9	5.42	19.4
Emulsion + serum + buffer......	5.44	43.9	5.48	30.8	5.48	24.0	5.44	19.7
Suspension alone	3.80	−21.7	3.80	−26.2	4.92	−35.4	3.33	0
Emulsion alone	3.34	−30.5	4.09	−28.2	3.69	−31.7	3.23	−27.3

	Beef Liver 5		Beef Liver 6		Cat liver			
Suspension + serum + buffer......	5.43	31.6	5.28	38.9	4.71	28.9		
Centrifuged suspension + serum + buffer	5.39	27.9	5.41	34.2	5.41	32.9		
Emulsion + serum + buffer........	5.49	27.7	5.45	50.2	5.49	58.9		
Suspension alone.. .	3.54	−24.3						
Emulsion alone. . . .	3.62	−30.5	3.72	−22.8	4.53	−32.7		

* A minus sign indicates esterification instead of hydrolysis.

† Duplicate experiments agreed poorly.

‡ The buffer was made with 2 N instead of 1 N HCl (see text).

included in the series. In five of the seven experiments (Table III) there was more hydrolysis with the suspensions than with the centrifuged extracts but the differences were small and it is impossible to conclude that any one of the three preparations studied (suspensions, extracts, and emulsions) was superior to the

others. In no instance were values found of the magnitude reported by Klein. An esterification of cholesterol occurred in emulsions incubated alone (see the preceding section), and the same reaction took place to about the same degree (except with Beef Liver 4) in the saline suspensions.

The buffer employed by Klein (2.0 cc. of 2 N sodium acetate, 1.4 cc. of 2 N HCl, and 0.6 cc. of water) has a pH of 4.2 and in mixtures with liver extract and serum in the proportion stated it gives a pH of about 4.7. A buffer made as described by Klein with the substitution of 1 N for 2 N HCl gives a pH of about 5.3 in the mixtures and, since Klein referred throughout to a pH of 5.3 as that of maximal hydrolysis, we used this buffer in our work. Because of our failure to obtain the high degree of hydrolysis reported by Klein, a buffer made as described by him with 2 N HCl (acid buffer (Table III)) was employed in two of the foregoing experiments. In one (Beef Liver 4) the hydrolysis was increased at the more acid pH; in the other (Beef Liver 1), though the result was not conclusive, there was no evidence for a considerably smaller degree of hydrolysis at pH 4.7, as would be expected from the curve published by Klein. Hence, suspensions of three rat livers were incubated with the buffers made with 1 N and 2 N HCl, respectively. The hydrolysis was approximately twice as great with the more acid buffer.

Saline Extracts of Brain—Extracts of rat brain, prepared according to Klein and centrifuged, did not hydrolyze cholesterol esters of blood serum in experiments like those reported with liver extracts in Table II.

Glycerol Extracts of Liver and Brain—No appreciable change in the ratio of combined to free cholesterol occurred on incubation of glycerol extracts of rat liver and brain with blood serum.

Experiments with Tissue Slices. Liver—Liver slices were immersed in Tyrode's solution and incubated for 1 or 2 days. The results were inconsistent, showing a small but significant esterification of cholesterol in two of seven experiments and no change, or questionable hydrolysis of cholesterol esters, in the others. No evidence for the presence of an active cholesterol esterase was obtained in experiments in which liver slices were incubated in blood serum.

Brain—No formation of cholesterol esters occurred in brain

slices incubated in Tyrode's solution or blood serum. The esterification of free cholesterol proceeded normally in the serum in the presence of the slices.

Enzymatic Esterification of Cholesterol in Carbon Tetrachloride— Sym in a series of papers which have attracted little attention reported marked enzymatic esterification in non-aqueous media. Acids and alcohols of low molecular weight were employed in most of his experiments, but brief reference is made (23) to the esterification of cholesterol with butyric acid or oleic acid in carbon tetrachloride solution in the presence of a dried pancreas preparation and bile salt. This finding was confirmed in experiments of which the following is typical.

To 1 cc. of a solution of cholesterol (0.5 M) and of butyric acid (0.5 M) in carbon tetrachloride were added 80 mg. of dried, defatted pancreas, prepared according to Sym (10), and 0.1 cc. of a 15 per cent solution of sodium taurocholate in water. The mixture was sealed in a small test-tube and agitated by rotation on a wheel in the incubator (37–38°) for 3 days. 65.2 per cent of the cholesterol was esterified. The same result was obtained with glycocholate instead of taurocholate. The taurocholate was a Hoffmann-La Roche preparation of satisfactory purity according to the criteria of Cortese and Bashour (24). The glycocholate was purified according to Cortese's directions.[3] It was necessary to dilute the solutions 2500 times for analysis by our method. Considerable error was thus introduced in the total cholesterol determination; but this made no difference in the interpretation of the results, since, as in all of this work, calculations were based on the ratio of combined to free cholesterol.

In experiments like that described cholesterol was about 30 per cent esterified with palmitic acid and about 40 per cent with oleic acid. . No hydrolysis of cholesterol palmitate occurred under the same conditions. With minced fresh pancreas instead of the dried preparation there was no esterification of cholesterol with butyric acid or hydrolysis of cholesterol palmitate.

Our hope that the Sym procedure would be a valuable tool in studying cholesterol esterase in tissues other than pancreas (25) was not realized. Dried, defatted powders were prepared from

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[3] We are indebted to Dr. Frank Cortese for much helpful advice in the purification of bile salts.

rat and cat livers and brains with the procedure used in making the pancreatin, and substituted for pancreatin in experiments like the one described. There was no esterification even when the amount of dried tissue was increased from 80 to 400 mg. The same result was obtained with sodium glycocholate. The preparations did not hydrolyze cholesterol palmitate.

SUMMARY

1. The presence in liver of a cholesterol-esterifying system, active over a wide pH range on the acid side of neutrality, was demonstrated by incubating emulsions of liver made in water or suspensions made by grinding with sand and physiological saline. The factor was shown to be different from the esterifying enzyme of blood serum; the reaction took place in blood-free preparations and there was no esterification of cholesterol in serum in the acid pH range in which the liver esterification occurs.

2. The same emulsions and suspensions caused hydrolysis of cholesterol esters when incubated with blood serum.

3. Cholesterol was esterified with fatty acids on incubation in carbon tetrachloride solution in the presence of acetone-extracted, dried pancreas and sodium taurocholate or glycocholate. No hydrolysis of cholesterol palmitate occurred in similar experiments. Under the same conditions preparations from liver showed no cholesterol esterase activity.

4. No evidence for the presence of cholesterol esterase in brain was obtained in similar studies.

5. The bearing of the results on the hypothesis that fatty acids are transported as cholesterol esters is discussed.

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ENZYMATIC HYDROLYSIS OF *d*-PEPTIDES*

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For many years it was generally believed that peptidases were specific for one optical form of the substrate which they attacked. The reports of Kögl and Erxleben (1) concerning the existence of *d*-amino acids in tumor proteins stimulated the search for proteolytic enzymes possessing the ability to hydrolyze substrates containing *d*-amino acids.

d-Peptidases have been reported to occur in the blood serum of humans with cancer by Waldschmidt-Leitz *et al.* (2, 3) but other workers (4–6) have obtained negative results. Hydrolyses of *d*-peptides by extracts of tumor tissue have been reported in some cases (4, 7) but not in others (8). It has also been claimed that *d*-peptidases can be produced in the blood of rats by the injection of racemic dipeptides (3).

We have been unable to repeat the observations of Waldschmidt-Leitz and Mayer (2) concerning the equal rate of hydrolysis of the *d* and *l* forms of leucylglycine by human cancer sera, or the artificial production of any considerable amount of *d*-peptidases in blood sera of rats by the injection of a racemic dipeptide (3). Both normal and cancer sera appear to be able to hydrolyze *d*-peptides very slowly, but this is not particularly significant, since many normal and cancer tissue peptidase systems can also do the same.

While tissue extracts from animal sources have been found to hydrolyze *d*-leucyl peptides only slowly, extracts from some bac-

teria have the ability to split the d form almost as rapidly as the l form. The presence of d-peptidases is therefore not characteristic of tumor growth. Furthermore, previous theories of the optical specificity of certain peptidases (9, 10) cannot be assumed to be applicable to all types of peptidases.

Methods

Blood serum was obtained from rats by centrifuging clotted blood immediately after withdrawal from the animal. Human cancer serum was prepared by centrifuging clotted samples of blood obtained from patients suffering from stomach cancers.[1] A peptidase extract from Flexner-Jobling rat carcinoma tissue was made as follows: active carcinoma tissue was carefully separated from normal and necrotic tissue, ground with sand, suspended in 2.5 times its weight of aqueous glycerol (20 per cent by volume), and allowed to extract without pH adjustment for 20 hours at 2°. The extract was clarified by centrifugation.

Injections of racemic peptide were carried out as follows: 1 cc. of 3 per cent dl-leucylglycine was injected into the tail vein of each rat three times weekly; at various intervals, one or two animals were sacrificed for serum analyses. A group of mice was injected subcutaneously with 0.2 cc. of 3 per cent dl-leucylglycine per mouse three times weekly, and again analyses for d-peptidases in the serum were made at intervals.

Peptidase preparations were obtained from *Bacillus megatherium*, *Clostridium butylicum*, *Leuconostoc mesenteroides*, and *Pseudomonas fluorescens* by autolysis of cells which had been alternately frozen and thawed. *Bacillus megatherium* was grown as previously described (11). *Clostridium butylicum* was grown on a 1 per cent glucose, 0.5 per cent tryptone medium for 24 hours at 37°. A medium was used for *Leuconostoc mesenteroides* which gave 5 times the enzyme and cell yield previously obtained (12). It consisted of 3 per cent malt sprouts extract, 1 per cent solubilized liver (13), 1 per cent glucose, and 1.5 per cent of hydrated sodium acetate. The organism was grown in this medium for 24 hours at 37° and then separated out by centrifugation. *Pseudomonas fluorescens* was grown for 42 hours at 30° with aeration in a medium

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[1] We are indebted to Dr. H. P. Rusch of the McArdle Memorial Laboratory, University of Wisconsin, for these samples.

containing inorganic salts, 0.05 M glucose, and 0.02 M ammonium chloride.

For hog erepsin, a fresh aqueous extract of hog intestinal mucosa was used; for chick erepsin, an aqueous glycerol (10 per cent glycerol by volume) extract of intestinal mucosa was used. Peptidase preparations were made from dried ground malt by aqueous extraction and from brewers' yeast by collecting the autolysis juice from pressed yeast which had been frozen and thawed alternately. This juice was dialyzed 4 hours before use.

Details concerning the substrates and methods used to determine the activity of the enzyme preparations have been given in a previous paper (14). Racemic substrates were always present in M/15 concentration and others in M/30 concentration. The pH was 8.0 ± 0.2 in all cases. In the case of dl mixtures, per cent hydrolysis of one linkage of one component is indicated. Experimental values have been corrected for enzyme blanks when this was necessary. Toluene was added as a preservative when the hydrolysis time exceeded 8 hours.

Results

In Table I are included the data obtained with one sample of serum from a human patient with advanced gastric cancer. Essentially the same results were obtained with sera from two other patients with stomach cancer. It may be seen that the dl forms of the leucyl peptides were hydrolyzed slowly, but no hydrolysis of the d forms could be detected. The peptidase activity varied as much as 3-fold in the three samples, but such differences are not peculiarly characteristic of tumor growth (6).

Sera from normal rats and rats with Flexner-Jobling carcinoma have been investigated, but, again, d-leucylglycine was split extremely slowly; the dl form was split at least 300 times as rapidly.

In the case of the extract from rat carcinoma tissue, no detectable hydrolysis of d-leucylglycine was obtained unless an activator such as Mn ions was present. The dl-peptide was hydrolyzed 135 times as rapidly as the d form.

Sera from rats injected in the tail vein with dl-leucylglycine were analyzed after three, seven, nine, twelve, and twenty-one injections, but in no case was d-leucylglycine or d-leucyldiglycine hydrolyzed more rapidly than at one-twentieth the rate of the dl forms; the usual rate was very much slower than this.

A pooled serum sample from seven mice which had received seven subcutaneous injections of *dl*-leucylglycine hydrolyzed *dl*-leucylglycine at least 55 times as rapidly as the *d* form.

TABLE I

d-Peptidase Activity of Serum and Tumor Preparations

Source	Substrate	Enzyme in 3 cc. reaction mixture	Time of incubation	Hydrolysis No activator	Hydrolysis 0.001 M MnSO₄
		cc.	*hrs.*	*per cent*	*per cent*
Human cancer serum	*dl*-Leucylglycine	0.5	21	16	22
	d-Leucylglycine	0.75	21	0	−4
	dl-Leucyldiglycine	0.5	8	20	26
	d-Leucyldiglycine	0.5	22	−2	
Normal rat serum	*dl*-Leucylglycine	0.75	0.75	30	64
	d-Leucylglycine	0.75	20		0
Rat tumor "	*dl*-Leucylglycine	0.75	0.75	40	70
	d-Leucylglycine	0.75	20	4	6
" carcinoma tissue	*dl*-Leucylglycine	0.10	1	20	30
	d-Leucylglycine	0.75	18	0	30

TABLE II

Hydrolysis of d-Leucylglycine by Various Peptidase Systems

Source	Substrate	Enzyme in 3 cc. reaction mixture	Time of incubation	Hydrolysis No activator	Hydrolysis 0.001 M MnSO₄	Hydrolysis Mn + cysteine*	Relative activity ratio, dl/d
		cc.	*hrs.*	*per cent*	*per cent*	*per cent*	
Malt	*dl*-Leucylglycine	0.75	1	44	50	60	
	d-Leucylglycine	0.75	23	6	27	43	32
Brewers' yeast	*dl*-Leucylglycine	0.10	0.5			78	
	d-Leucylglycine	0.40	3	5		58	32
Hog erepsin	*dl*-Leucylglycine	0.10	0.5			45	
	d-Leucylglycine	0.75	3	0	8	34	65
Chick erepsin	*dl*-Leucylglycine	0.03	0.67	40	28		
	d-Leucylglycine	0.60	1.5	6	74	80	23

* 0.001 M MnSO₄ plus 0.003 M cysteine.

TABLE III

Hydrolysis of d-Peptides by Bacterial Peptidases

Source	Substrate	Enzyme in 3 cc. reaction mixture	Time of incubation	Hydrolysis			Relative activity ratio, *dl/d*
				No activator	0.001 M MnSO₄	Mn + cysteine*	
		cc.	hrs.	per cent	per cent	per cent	
Bacillus mega-therium	dl-Leucylglycine	0.3	0.5	0	0	20	
	d-Leucylglycine	0.5	1.0	0	0	47	1.4
	dl-Leucyldiglycine	0.2	0.5	5	31	34	
	d-Leucyldiglycine	0.75	3.0	0		26	29
Clostridium bu-tylicum	dl-Leucylglycine	0.3	0.5	8	25†	92	
	d-Leucylglycine	0.75	2.0	2	48†	40	23
	dl-Leucyldiglycine	0.10	1.0	4	24	62	
	d-Leucyldiglycine	0.10	2.5	4		30	5.2
Leuconostoc mes-enteroides	dl-Leucylglycine	0.20	1.0	30	42†	22	
	d-Leucylglycine	0.75	13	3	56†	16	36
	dl-Leucyldiglycine	0.14	0.5	38	32	37	
	d-Leucyldiglycine	0.30	0.5	36		16	2.2
Pseudomonas flu-orescens	dl-Leucylglycine	0.20	1.0	52	78	49†	
	d-Leucylglycine	0.80	1.0	3	0	20†	16

* 0.001 M MnSO₄ plus 0.003 M cysteine.

† 0.001 M CoCl₂ was present instead of MnSO₄ or Mn plus cysteine.
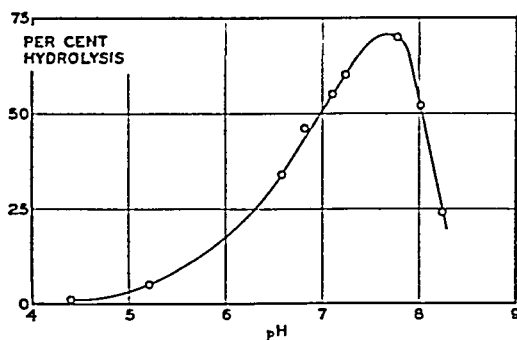
FIG. 1. Effect of pH on hydrolysis of *d*-leucyldiglycine by *Leuconostoc mesenteroides* cell autolysate.

The slow hydrolysis of *d*-leucylglycine by various sera is not at all remarkable, since many peptidase systems can split this peptide slowly, as shown in Table II. It may be seen that peptidases of malt, hog intestinal erepsin, chick erepsin, and yeast are all capable of hydrolyzing *d*-leucylglycine about one-thirtieth to one-sixtieth as rapidly as the *dl* form, provided suitable activators are present. Whereas peptidases from tumor sera and normal and tumor tissues do not appear to possess any unusual powers of splitting *d*-peptides, enzyme preparations from some microorganisms do have the ability to hydrolyze *d*-leucyl peptides almost as rapidly as the *l* forms. This is illustrated in Table III. *Bacillus megatherium* peptidases hydrolyze *d*-leucylglycine very rapidly and *Leuconostoc mesenteroides* and *Clostridium butylicum* peptidases hydrolyze *d*-leucyldiglycine very rapidly. In all cases except the splitting of *d*-leucyldiglycine by *Leuconostoc mesenteroides*, activators are necessary for maximum rates of hydrolysis.

In the case of *Leuconostoc mesenteroides*, the pH optimum for the hydrolysis of *d*-leucyldiglycine has been found to be at 7.8 (Fig. 1). Preliminary attempts to separate the enzymes splitting the *l* and *d* forms of leucyldiglycine have not been successful, so that the number of enzymes involved cannot be stated.

SUMMARY

1. Sera from rats with Flexner-Jobling carcinoma and from humans with gastric cancer were unable to hydrolyze *d*-leucyl peptides at any appreciable rate, but were able to split *dl*-leucyl peptides.

2. Significant amounts of *d*-peptidases could not be artificially produced in rat serum by repeated injection of *dl*-leucylglycine into the tail vein. Subcutaneous injections of the same racemic peptide into mice also failed to produce *d*-peptidases in any amount in mouse serum.

3. Peptidases from chick mucosa, yeast autolysate, and malt are able to split *d*-leucylglycine about one-thirtieth as rapidly as *dl*-leucylglycine in the presence of suitable activators such as Mn or Mn-cysteine.

4. Peptidases from *Leuconostoc mesenteroides* and *Clostridium butylicum* are able to hydrolyze *d*-leucyldiglycine at least one-fifth to one-half as rapidly as *dl*-leucyldiglycine. Peptidases from

Bacillus megatherium and *Pseudomonas fluorescens* hydrolyze *dl*-leucylglycine only 2 to 16 times as fast as *d*-leucylglycine.

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A SIMPLE METHOD FOR THE QUANTITATIVE DETERMINATION OF PREGNANEDIOL IN HUMAN URINE

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Since the discovery by Venning and Browne (1) of sodium pregnanediol glucuronidate in human urine and the perfection of a quantitative method for its determination by Venning (2) this compound has received wide attention because of its close relation to the metabolism of the corpus luteum hormone. This subject has recently been reviewed by Marker and Hartman (3). It is apparent from conflicting results in the literature that difficulties have been encountered in measuring the excretion of the sodium pregnanediol glucuronidate complex. During the menstrual cycle, when only small amounts of the material are present in the urine, the identity of the final product is sometimes questionable. Furthermore, spontaneous hydrolysis of the complex to free pregnanediol during the period of collection may occur. The resulting inconstant recoveries are particularly noticeable in hot weather, when refrigeration is not available. This latter difficulty was met by Bucher and Geschickter (4) who, using a modification of Hartmann and Locher's (5) method as developed by Weil (6), were able to recover the free pregnanediol from the acetone used in the Venning procedure. The total excretion was estimated by adding this moiety to that recovered in the combined form. However, wide application of such methods is limited by the technical difficulties involved. With these factors in mind, a procedure has been developed which is sufficiently simple and accurate that it may be applied to routine clinical use. It involves three steps: (1) liberation of free pregnanediol by acid hydrolysis of the urine in the presence of toluene, (2) the separation of most of the impurities from the toluene extract by the precipitation with alcoholic

alkali, and (3) the final quantitative crystallization of the pure pregnanediol from aqueous alcohol.

Method

Urine specimens are collected, with a few drops of toluene as a preservative. The entire 24 or 48 hour volume or a suitable aliquot thereof is used. As 1 liter is a convenient amount, the figures given in the following steps are for this volume of urine; proportional amounts are used with other volumes. In some non-pregnancy urines, when very small amounts of pregnanediol are to be determined, 2 or 3 liter volumes should be taken, while with late pregnancy urine 50 to 500 cc. volumes are suitable.

1 liter of urine is placed in a 2 liter round bottom flask and 50 cc. of toluene are added. A wide bore reflux condenser, fitted with a cork stopper or ground glass connection, is attached and the urine heated to boiling. 100 cc. of concentrated hydrochloric acid (sp. gr. 1.19) are added slowly through the condenser and boiling continued for 15 minutes. The temperature of this boiling mixture is 100–105°. The flask is then cautiously removed to a vessel of cold water (violent boiling may occur if the hot mixture is shaken); the urine is brought to room temperature and transferred to a separatory funnel. The toluene layer, together with the layer of urine-toluene emulsion, is removed and the urine layer is reextracted twice with 25 cc. portions of toluene. The combined toluene extracts and emulsions are filtered through a Buchner funnel, with gentle suction, and the residue thoroughly washed with small amounts of toluene. If the filtration is performed without excessive suction, the emulsion is completely broken down. The filtrate is poured into a small separatory funnel, the urine layer is discarded, and the toluene extract is washed once with 50 cc. of water. The toluene is now transferred to a dry 400 cc. beaker, care being taken that no droplets of water are included, and the extract boiled on an electric hot-plate until the last traces of water are dispelled. 10 cc. of a 2 per cent solution of sodium hydroxide in absolute methyl alcohol are added and boiling continued until all the alcohol is driven off and the quantity of toluene is reduced to half its original volume. After the extract is allowed to stand at room temperature until cool, it is filtered with gentle suction into a 125 cc. Erlenmeyer flask

and the abundant gelatinous precipitate is thoroughly washed with three successive small amounts of toluene. The filtrate should be clear and free of all traces of a pink or brown color; otherwise the previous step must be repeated. The clear, pale greenish yellow filtrate is now taken to dryness on the hot-plate, the last traces of toluene being carefully removed by a stream of air to avoid overheating the residue. 10 cc. of 95 per cent ethyl alcohol are added and the residue is completely dissolved by heating. While heating is continued, 40 cc. of hot aqueous 0.1 N sodium hydroxide are added slowly. The stoppered flask is allowed to stand at room temperature until cool and is then placed in the refrigerator overnight.

The precipitate is collected by filtration, washed with water, dissolved in 10 cc. of alcohol, and precipitated a second time in the same manner, 40 cc. of distilled water being used instead of the sodium hydroxide solution. The pregnanediol should now be colorless and entirely crystalline; if not, one further precipitation from aqueous alcohol is carried out. The purified substance is now collected by filtration, transferred by means of ethyl alcohol to a tared vial, dried in an oven at 90°, and weighed. A melting point is taken on each sample recovered and, if this be below 220°, a mixed melting point is determined with a pure sample of pregnanediol. When the recovered material is largely pure pregnanediol, its melting point is raised when it is mixed with an authentic sample; the determination is considered to be satisfactory if there is no depression of the mixed melting point.

<div style="text-align:center">EXPERIMENTAL</div>

Precipitation of Pregnanediol from Aqueous Alcohol—In order to estimate the amount of pregnanediol which might be lost by remaining in solution, the solubility of pregnanediol in various concentrations of aqueous alcohol was determined in the following manner. Known amounts of pregnanediol in 95 per cent ethyl alcohol were diluted with water while hot. The solutions were cooled and placed in the refrigerator overnight. By preparing a series of such solutions with decreasing amounts of pregnanediol, it was possible to determine roughly how much was needed in order to give a precipitate. Such an experiment is shown in Table I, from which it may be seen that within the accuracy of

this method pregnanediol is completely insoluble when 3 or more volumes of water are added to 1 volume of alcohol. It would appear, therefore, that two or three precipitations from 10 cc. of alcohol, with 30 or more cc. of water, would result in no detectable loss. This is apparently true, also, in the presence of the other neutral substances present in the extract at this stage, as similar experiments were conducted in which cholesterol and pregnanediol-free neutral urine extracts were added to alcohol, together with known amounts of pregnanediol. No increased solubility of pregnanediol was noted in the presence of these substances in the amounts used. It appears probable, however, that small losses could occur at this step in the presence of large quantities of other lipids.

TABLE I

Approximate Solubilities of Pregnanediol in Aqueous Alcohol

95 per cent alcohol, cc.	10	10	10	10	10	10
Water added, cc.......	10	20	25	30	40	50
	mg.	mg.	mg.	mg.	mg.	mg.
Pregnanediol in solution						
Room temperature........	>1.0	1.0	0.25	<0.25	<0.25	<0.25
After refrigeration.......	>1.0	0.5	0.25	<0.04	<0.04	<0.04

It was of interest to determine the fate of other substances present in the neutral fraction when this precipitation was carried out, for one might expect that other urinary constituents may behave in a fashion sufficiently similar to pregnanediol to be present in the final product. The three most abundant neutral lipids known to be present in human urine were studied. These were cholesterol, androsterone, and a hydrocarbon designated by Hart and Northrup (7) and by Marker (8) as heptacosane. Cholesterol in amounts up to 8 mg. and the hydrocarbon in amounts up to 100 mg. were found to form stable colloidal solutions when precipitated in the above manner from 50 cc. of 19 per cent alcohol. On filtration these colloidal suspensions leave no residue. When present in larger amounts, some residue is retained on the filter. Androsterone separates as plate-like crystals from 50 cc. of the 19 per cent alcohol when present in amounts exceeding 4 mg.; amounts smaller than this remain in solution. Therefore, an excess of

16 mg. of cholesterol, 200 mg. of the hydrocarbon, or 8 mg. of androsterone per liter of urine would be necessary in order to interfere with the final purity of the pregnanediol, if the two precipitations from aqueous alcohol are carried out. These amounts are well outside the range of values thus far reported for these neutral substances in normal human urine.

Precipitation of Impurities from Toluene Extract—It is of importance that excess water be avoided in handling the toluene extract. Water in excess of the small amount resulting from chemical reaction may introduce impurities which are not removed by the subsequent step. The presence of water also results in a large gelatinous precipitate which is difficult to wash. Excess hydrochloric acid is removed by washing the original toluene extract, thus minimizing the water of reaction. Dissolved water is then largely dispelled by boiling.[1] The 2 per cent sodium hydroxide solution in methyl alcohol is prepared from clean dry sodium hydroxide pellets and absolute methyl alcohol. An alternative to the alcoholic sodium hydroxide solution is a 2 per cent methyl alcohol solution of sodium methylate, prepared by adding sodium to absolute methyl alcohol. Such a solution has been used with satisfactory results.

It seemed possible that some loss at this stage, through adsorption of the pregnanediol on the abundant precipitate, might be incurred. Therefore, precipitates from several batches of urine extract were combined and thoroughly extracted with boiling ethyl alcohol. After the filtered alcohol extract was evaporated off, the residue was taken up in toluene and the toluene-soluble material purified in the usual manner. No pregnanediol was recovered by this method from fourteen combined precipitates. Apparently, therefore, the loss of pregnanediol in this stage of purification is negligible.

Extraction of Pregnanediol from Urine by Toluene—1 liter volumes of urine from which 5 to 80 mg. of pregnanediol had been recovered by the extraction method described were exhaustively reextracted with toluene. In no case was any further pregnanediol recovered. In five instances the three toluene extracts described

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[1] An occasional difficulty in avoiding excess water may be met by the addition of 0.5 to 1 gm. of powdered calcium oxide to the boiling extract before cooling.

in the original procedure were worked up separately. The recoveries indicate that approximately 85 per cent of the free pregnanediol is taken up by the toluene during hydrolysis; the remainder usually appears in the second extraction, while only occasionally does the third extract contain a further trace. The volumes of toluene used were therefore considered sufficiently large to remove all available pregnanediol. Inasmuch as the solubility of pure pregnanediol in toluene at room temperature does not exceed 2 mg. per cc., no attempts were made to use smaller volumes, for fear of losses in subsequent steps.

Hydrolysis of Combined Pregnanediol by Acid—The rate of hydrolysis of the sodium pregnanediol glucuronidate complex was studied by adding measured amounts of an aqueous sodium pregnanediol glucuronidate solution to male urine and boiling for various periods of time in the presence of different concentrations of hydrochloric acid. The amount of free pregnanediol liberated was then determined by the method outlined above.

The first series of hydrolyses was made on 100 cc. samples of male urine containing 10 mg. of sodium pregnanediol glucuronidate. To this volume of urine amounts of concentrated hydrochloric acid from 5 to 15 volumes per cent were added; the mixtures were boiled for periods of $2\frac{1}{2}$ to 60 minutes. The amounts of free pregnanediol recovered are plotted in Fig. 1, from which it can be seen that with 5 volumes per cent of acid the hydrolysis is slow and that a maximal yield of 66 per cent was obtained after 1 hour's boiling. When 10 or 15 volumes per cent were used, a maximal yield of 70 per cent was obtained in $2\frac{1}{2}$ and 10 minutes respectively, but with longer boiling smaller amounts were obtained. It appeared from this that 10 volumes per cent of acid would give satisfactory results if destruction of the compound could be avoided. Consequently, a second series of hydrolyses was made on 1000 cc. volumes of urine to which 0.05 volume of toluene was added. In Fig. 2 are shown the results of such an experiment. The 10 volumes per cent of acid gave maximal yields within 10 minutes and prolonged boiling for 4 hours resulted in little destruction of the compound.

A third series of hydrolyses was made on 50 mg. amounts of combined pregnanediol in 1000 cc. of distilled water to which were added 50 cc. of toluene and 100 cc. of concentrated hydrochloric

acid. A maximal yield of 80 per cent was obtained after 15 minutes of boiling and continued boiling for 4 hours resulted in only slight destruction. In Fig. 2 these results are compared with similar experiments in which no toluene was used. It is apparent that the presence of toluene serves to prevent destruction of pregnanediol by the acid.
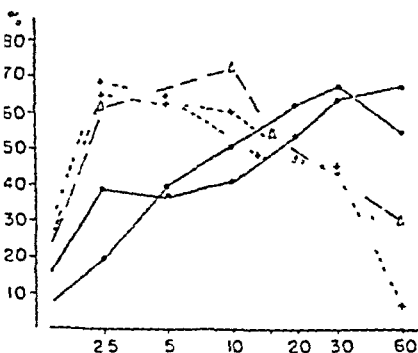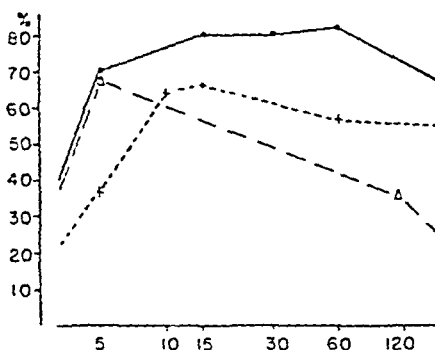
FIG. 1.   FIG. 2.

FIG. 1. Liberation of free pregnanediol from 10 mg of sodium pregnanediol glucuronidate on boiling with acid in 100 cc. of normal male urine. The dots represent 5, the crosses 10, and the triangles 15 volumes per cent of concentrated hydrochloric acid. The ordinate represents per cent of pregnanediol recovered; the abscissa, time of boiling in minutes (logarithmic scale).

FIG. 2. Hydrolysis, in 1000 cc. of an aqueous solution, of 50 mg. amounts of sodium pregnanediol glucuronidate with 10 volumes per cent of concentrated hydrochloric acid. The dots represent results with, the triangles without 50 cc. of toluene. The crosses show the rate of hydrolysis in 1000 cc. of male urine with 100 cc. of concentrated hydrochloric acid and 50 cc. of toluene.

Recoveries of Pregnanediol by Method Proposed—Amounts of sodium pregnanediol glucuronidate from 2 to 200 mg. were added to samples of urine from normal males. The volumes of urine used were 1000, 2000, and 4000 cc.; these were treated by the above method and the yields calculated. The results are given in Table II, which shows the calculated equivalent of free pregnanediol contained in the amount of the complex added, the amount recovered, and the per cent recovery.

Table II shows that yields from 1 liter of urine containing 3 mg.

TABLE II

Recovery of Pregnanediol

Urine volume	Sodium pregnanediol glucuronidate added	Calculated equivalent of free pregnanediol*	Free pregnanediol recovered		Deviation from mean recovery of 68.02 per cent
cc.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
1000	0	0	0		
	0	0	0.8		
	2	1.236	0.0		
	2	1.236	0.0		
	2	1.236	0.5	41.5	
	2	1.236	0.6	50.6	
	2	1.236	1.2	100.0	
	5	3.10	2.3	74.2	+6.18
	5	3.10	2.3	74.2	+6.18
	5	3.10	2.4	77.4	+9.38
	5	3.10	2.5	80.6	+12.58
	10	6.18	3.9	63.1	−4.92
	10	6.18	4.0	64.7	−3.32
	10	6.18	4.1	66.4	−1.62
	16	9.89	5.9	60.0	−8.02
	20	12.36	8.2	66.4	−1.62
	20	12.36	8.4	68.0	−0.02
	20	12.36	8.7	70.4	+2.58
	25	15.45	9.5	61.5	−6.52
	30	18.54	12.0	64.8	−3.22
	30	18.54	12.4	67.0	−1.02
	30	18.54	13.3	71.8	+3.78
	40	24.72	16.5	66.8	−1.22
	40	24.72	16.5	66.8	−1.22
	50	30.90	19.9	64.4	−1.62
	50	30.90	20.0	64.7	−3.62
	50	30.90	21.7	70.3	+2.28
	200	123.60	80.6	65.2	−2.82
2000	0	0	0.5		
	2	1.236	0.0		
	2	1.236	0.0		
	2	1.236	0.1	8.33	
	2	1.236	1.1	91.66	
	2	1.236	1.4	116.66	
	5	3.10	1.1	35.48	
	5	3.10	1.6	51.61	
	5	3.10	1.7	54.83	
4000	0	0	0.0		
	0	0	0.1		
	2	1.236	1.7	141.66	
	5	3.10	0 6	19.35	
	5	3.10	1.0	32.66	
	5	3.10	2.2	70.96	
	10	6.18	3.2	53.2	
8000	0	0	0.0		

* Calculated with the conversion factor of 0.618.

or more of pregnanediol are consistent and average 68 per cent. The standard deviation from the mean was 5.15 per cent and the standard error of the mean was 1.12 per cent. Recoveries of smaller amounts of pregnanediol were less uniform, but some quantitative significance could be attached to determinations of 1 to 3 mg. per liter, when 2 or 4 liter volumes were used.

Similar volumes of male urine to which no sodium pregnanediol glucuronidate was added were hydrolyzed and extracted in the same manner. Two of these samples yielded no precipitate at the final step, while three gave readings of 0.1 to 0.8 mg. per liter.

The melting points of the recovered material in the determinations shown in Table II ranged from 218–228°, and on mixing with pure pregnanediol (m.p. 238°) no depression occurred.

Pregnanediol determinations on human urine during the luteal phase of the menstrual cycle and during pregnancy have yielded material with melting points ranging from 205–228°. These melting points were not depressed and usually were elevated when the material was mixed with the pure substance. The lower values are apparently due to a small amount of unidentified material not present in hydrolysates of the combined form. The possibility that this impurity is allopregnanediol has not been eliminated.

DISCUSSION

The calculation used in determining the free pregnanediol equivalent of sodium pregnanediol glucuronidate may be subject to error. Venning and Browne showed that sodium pregnanediol glucuronidate contains 1 molecule of water of crystallization and has a molecular weight of 536. As the molecular weight of free pregnanediol is 320, these authors used a factor of 0.597 for the conversion of the complex to free pregnanediol. Bachman, Leekley, and Hirschmann (9) found that the combined form is hygroscopic, but that all water is removed when the material is dried in a vacuum over phosphorus pentoxide at room temperature. It has been assumed in the above calculations that no solvent of crystallization was present in the sodium pregnanediol glucuronidate used, and that 1 gm. of the complex contained 0.618 gm. of free pregnanediol. The material used was repeatedly recrystallized from 95 per cent ethyl alcohol and dried in air at 90°.

It appears unlikely that the failure to obtain 100 per cent re-

covery on hydrolysis of the complex in aqueous solution can be attributed to an error in this calculation. However, the consistent loss of 20 per cent under varied conditions cannot be traced to incomplete extraction and, as destruction is largely eliminated by the use of toluene, it remains unexplained. The further loss in determinations on urine is believed to be incurred in purification. The recoveries given in Table II show that determinations on urine containing 3 mg. or more of pregnanediol per liter may be made with considerable accuracy. When 1 to 3 mg. of pregnanediol per liter is present, the error involved is large, but this error is somewhat reduced by increasing the volume of urine extracted.

On two occasions no pregnanediol was detected in 1 liter samples of urine containing 1.2 mg. of pregnanediol. Some samples of normal male urine yielded zero values, while others gave 0.1 to 0.8 mg. of some unidentified substance per liter of urine. It is assumed that the recovered material in these cases is not pregnanediol, but is an impurity which may lead to error in determinations on urine containing comparable amounts of pregnanediol. Although Engel, Thorn, and Lewis (10) isolated 64 mg. of pregnanediol from 1000 liters of normal male urine, the zero values shown in Table II indicate that amounts of pregnanediol of the order found by Engel, Thorn, and Lewis are not uniformly detectable by the above method.

Subject to these limitations the method proposed is satisfactory. It requires no special apparatus, it is free of technical difficulties, and no abnormal urinary constituents such as albumin or blood have been found to interfere. As pregnanediol is a stable substance, no special precautions are required in the collection of specimens and the loss through hydrolysis which is so inconstant in determination of the combined form is obviated.

To estimate the 24 hour excretion of pregnanediol the following approximate calculation is made

$$\text{Daily pregnanediol output, mg.} = \frac{24 \text{ hour urine volume}}{\text{volume urine used}}$$

$$\times \text{ pregnanediol found, mg.} \times 1.47$$

which takes into consideration the average loss entailed in the method. Although the correction factor seems large, it is over-

balanced by the uniformity of results, by the stability of free pregnanediol, and by the simplicity of the method.

The authors are indebted to Dr. L. L. Engel for valuable advice and criticism and to Dr. P. G. Weil for many helpful suggestions.

SUMMARY

The difficulties encountered in the determination of sodium pregnanediol glucuronidate are cited and a method based on the determination of the free form of pregnanediol is described.   The advantages and limitations of the method are discussed.

It is recommended as a satisfactory procedure for routine clinical use or for the large scale preparation of pregnanediol from human urine.

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THE EFFECT OF ALKALOSIS ON THE RELATIONSHIP BETWEEN SERUM CALCIUM AND PROTEIN IN VIVO*

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In the absence of definite experimental evidence, it has generally been assumed that the symptoms of tetany associated with increases in serum pH are due to a decrease in the concentration of ionic calcium. However, by means of the frog heart method, McLean and Hastings (1) were unable to demonstrate any significant change in calcium ions with change in pH. Because of the relatively narrow pH range in which these investigators worked, and the limited range of sensitivity of the method, no definite conclusions were offered, and the question of the effect of alkalosis on the capacity of protein to bind calcium was left open.

It has been abundantly confirmed that the concentrations of total calcium and protein in serum, in the absence of recognizable defects of calcium metabolism, bear a linear relationship to each other. This has been expressed by the regression equation, total Ca $= a \times$ total protein $+ b$, in which a and b are constants. Using data accumulated in the literature, McLean and Hastings (1) have demonstrated that the value for the intercept b in this equation is a close approximation of the concentration of ionic calcium as determined by their frog heart method, under normal conditions.

On the basis of these considerations, the relationship between total calcium and proteins in the serum of normal animals, and those with marked elevation of serum pH, was studied. It was hoped that by these means some idea would be gained as to the effect of alkalosis on the ability of serum protein to bind calcium

and the magnitude of the intercepts in the respective regression equations, which might give an approximation of the effect of alkalosis on the concentration of calcium ion.

Procedure and Methods

Cats were used as the experimental animals. They were fed on canned salmon and milk for at least 1 week before the experimental procedure. The method employed for producing alkalosis was as follows: The animals were injected intraperitoneally with a solution containing 50 gm. of glucose and 200 to 300 milliequivalents of sodium bicarbonate per liter, in amounts approximating 100 cc. per kilo of body weight. 5 hours later, a similar quantity of fluid was withdrawn. The serum changes resulting from this procedure have, in part, been previously described (2). These were an increase in pH and total CO_2, a decrease in chloride, and relatively little change in sodium. These changes persisted practically unchanged for at least 24 hours. At intervals of 2 hours, beginning with the withdrawal of the fluid from the peritoneal cavity, samples of blood were removed in amounts varying from 15 to 20 cc. A total of twenty-four blood samples was examined from six different animals. Of these, two animals survived 24 hours and were sacrificed, two animals died after 6 hours, and two animals died after 10 hours. The control observations were made on five animals. Of these, three animals were not treated in any way, and two animals were subjected to depletion of extracellular electrolytes as previously described (3). This resulted in marked hemoconcentration. By this procedure, and by the frequent withdrawal of fairly large quantities of blood, an adequate distribution of serum protein concentrations was obtained. A total of twenty-nine observations was made in the control group. All manipulations were carried out under nembutal anesthesia.

The blood was collected and allowed to clot under oil. The chemical analyses were carried out on the serum which was removed in the usual manner. Total protein was calculated from the nitrogen content determined by the Kjeldahl procedure. Total CO_2 was determined by the method of Van Slyke and Neill (4), phosphate by the method of Fiske and Subbarow (5), calcium by the method of Clark and Collip (6), and pH by Cullen's method (7) as modified for the Evelyn colorimeter.

Results

The data may be most conveniently presented by means of the statistical analysis. This is summarized in Table I. The average concentration of serum protein in both groups showed no significant difference. The range in the control animals was from 5.4 to 8.5 per cent, and in the alkalotic animals from 4.3 to 8.5 per cent. There was a significant decrease in the average calcium concentration in the alkalotic group, the individual values having a range from 6.3 to 10.4 mg. per cent. In the control group the calcium concentrations varied from 8.6 to 11.7 mg. per cent. There was a significant increase in the average value for phosphorus in the alkalotic group with a range in the individual values from 5.5 to 17.1 mg. per cent. In the control group phosphorus

TABLE I

Results of Serum Analyses in Normal and Alkalotic Animals

	No. of observations	Total protein	Ca	P	Total CO₂	pH
		gm. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	m.eq. per l.	
Control........	29	6.84	10.9	6.46	17.48	7.41
		±0.13*	±0.15	±0.38	±0.92	±0.02
Alkalotic......	24	7.08	7.98	10.29	40.58	7.65
		±0.19	±0.25	±0.60	±0.98	±0.02

* Standard error.

varied from 3.5 to 11.4 mg. per cent. The degree of alkalosis produced by the procedure employed may be seen from the average values for total CO_2 and pH.

Relationship between Protein and Calcium—The correlation coefficient between total protein and calcium in the serum of the normal animals was 0.55 ± 0.13, and the regression equation expressing this relationship Ca = 0.62 protein + 6.7, in which the standard error of the regression coefficient is 0.18, and of the intercept 0.12. This agrees fairly closely with similar equations obtained in humans (8–10) and in dogs (11).

In the animals with alkalosis the correlation coefficient between protein and calcium was 0.58 ± 0.17, and the regression equation Ca = 0.76 protein + 2.61, in which the standard error of the regression coefficient was 0.22, and of the intercept 0.20.

Relationship between Phosphorus and Calcium—In the control group no correlation could be demonstrated between calcium and phosphorus. In the animals with alkalosis the correlation coefficient was -0.34 ± 0.19, which statistically cannot be considered significant. When the data from both groups are combined, a significant negative correlation coefficient between calcium and phosphorus is obtained equal to -0.56 ± 0.10. The regression equation expressing this relationship was $Ca = -0.26 P + 11.3$, in which the standard error of the regression coefficient is 0.05, and of the intercept 0.17. The regression coefficient in this equation is very similar to that obtained by Peters and Eiserson (8) in human serum.

DISCUSSION

The type of alkalosis produced by the procedure employed in these experiments is essentially similar, as regards the resulting serum electrolyte configuration, to that following prolonged vomiting of gastric juice, or the administration of sodium bicarbonate to patients with impaired renal function. In the former, however, one frequently encounters reduced serum sodium concentrations as well. The effect of alkalosis on the concentration of serum calcium has been previously studied with equivocal results. Thus, Peters and Van Slyke (12) and Schmidt and Greenberg (13) in their reviews of this phase of calcium metabolism reach the conclusion that the alkalosis resulting from the administration of bicarbonate is not associated with a significant decrease in serum calcium. On the other hand, Stewart and Haldane (14), and recently McCance and Widdowson (15) in a case of pyloric obstruction, were able to demonstrate hypocalcemia, presumably secondary to an alkalosis due to excess alkali. Our experiments confirm the latter observations.

It will be noted in Table I that the variations in serum pH in both control and alkalotic groups are very small. In both groups about two-thirds of the individual observations are within 0.1 unit of the average for the group as a whole. The regression equations expressing the relationship between calcium and protein may therefore be considered as representing this relationship at two different but fairly constant levels of serum pH. A comparison of the two regression equations indicates: first, that the average

quantity of calcium bound to protein is slightly greater in the alkalosis group, although statistically this may not be significant; second, there is a significant decrease, in the alkalotic group, of calcium not combined to protein. This latter fraction of the total calcium represents primarily ionic calcium, although a small but appreciable quantity of bound but diffusible calcium is also included in this fraction (1).

The explanation for the reduction in ionic calcium during alkalosis is not clear. It will be noted that associated with this finding a significant hyperphosphatemia was demonstrated. This has been noted by others (15-17). Our data, however, add nothing directly bearing on the problem of the inverse relationship shown to exist between serum calcium and phosphorus under various conditions. The absence of a significant correlation between calcium and phosphorus in the individual groups is probably due to the lack of an adequate variation in serum phosphorus. When both groups were combined, the expected inverse relationship could be demonstrated.

McLean and Hastings (1), from their study of calcium ion concentrations in serum of various dilutions, concluded that the relationship of bound to ionic calcium follows the mass law equation describing the ionization of calcium proteinate. If it be assumed that [total Ca] $-$ [Ca^{++}] $=$ [calcium proteinate], and [total protein] $-$ [calcium proteinate] $=$ [protein$^-$], the mass law equation describing the ionization of calcium proteinate takes the following form

$$[\text{Total Ca}] = \frac{[\text{Ca}^{++}]}{[\text{Ca}^{++}] + K} [\text{total protein}] + [\text{Ca}^{++}]$$

As McLean and Hastings point out, the empirical calcium and protein regression equation is a special case of the mass law equation in which [Ca^{++}] represents the intercept, and [Ca^{++}]/([Ca^{++}] $+$ K), the slope. The rectilinearity of the regression equation is therefore dependent on the fact that, under the conditions in which the empirical regression equations cited in the literature were obtained, [Ca^{++}] tended to be relatively constant. This was demonstrated by McLean and Hastings who found, by actual determination of [Ca^{++}] in serum and protein containing body fluid containing widely varying concentrations of protein, a small

range in [Ca++] variability. Presumably, in our alkalosis experiments, the relatively constant value for serum pH was also associated with relatively little variability in [Ca++] in the group as a whole. From our data, we may calculate the pK of calcium proteinate for the control and alkalotic groups. The average values for calcium and protein are given in Table I, and expressed as moles per kilo of H_2O. The concentration of water is obtained from the formula, 99 − 0.75 protein. The factor for converting protein into base-combining equivalents was obtained from the data of Van Slyke, Hastings, Hiller, and Sendroy (18), with the average values for pH in Table I, and assuming an albumin to globulin ratio of 1.8 and a valence of 2 for the serum proteins. Calcium ion concentrations were obtained from the intercepts of the respective regression equations and expressed as moles per kilo of H_2O. In this fashion, pK values for calcium proteinate in the control and alkalotic groups were calculated as 1.96 and 2.37 respectively. In a similar manner, McLean and Hastings calculated the respective pK values from the calcium and protein regression equations given by five different investigators. These were found to vary from 1.97 to 2.28, averaging 2.12. The pK value for calcium proteinate determined by Hastings and McLean from their data on calcium ion concentrations in serum with the frog heart method was 2.22 ± 0.07. Considering the limited number of observations with the intercept in the regression equation as a measure of [Ca++], our values for the pK of calcium proteinate cannot be considered as significantly out of line. It must therefore be concluded, tentatively, that our data on the relationship between serum calcium and protein *in vivo*, in the presence of marked elevations of serum pH, agree with the conclusions of Hastings and McLean that the behavior of these serum constituents may be described by a simple mass law equation as a first approximation.

SUMMARY

The relationship between calcium and protein in the presence of a marked degree of alkalosis was studied in cats.

A significant hypocalcemia due presumably to marked reduction in calcium ion concentration was demonstrated. Hyperphosphatemia was also found.

The relationship between calcium and protein was such as to agree with the conclusion of McLean and Hastings that this relationship may be described, as a first approximation, by a simple mass law equation yielding the ionization constant of calcium proteinate.

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A NEW PHOTOMETRIC METHOD FOR THE
DETERMINATION OF IRON

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In the study of many types of biological material it has become of increasing importance to determine accurately extremely small amounts of iron. Since color reactions are, as a rule, very sensitive, colorimetric procedures are particularly useful in the microanalytical field, and several colorimetric methods have appeared in the literature on iron determination.

Although the extremely sensitive thiocyanate reaction is widely used for quantitative purposes, and has been the basis for much of our knowledge of iron concentration in biological material, the difficulties of the thiocyanate method have all combined to make investigators welcome the proposal of other processes, and pyrocatechol (1), pyramidon (2), thioglycolic acid (3), dimethylglyoxime (4), the H_2FeCl_5 complex (5), α,α'-bipyridine (6), 8-hydroxyquinoline (7), o-phenanthroline (8), ferron (9), etc., have successively been proposed for chromogenic agents, and applied to the quantitative determination of iron.

Lutz (10) has shown that acid solutions of ferric ions give a blue-green color with protocatechuic acid, and that ferrous ions give no color under the same conditions. The blue-green color formed by the interaction of ferric ion and protocatechuic acid changes to red in solutions made alkaline, and alkaline solutions of ferrous ions give a red color with the reagent, as does ferric ion under similar conditions. The extreme sensitivity of this color reaction induced the present author to adapt it into a workable and reliable procedure which has been found to be a simple, rapid, and accurate method for the determination of iron in biological materials.

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Method

Reagents—We use two reagents, which will be designated Reagents A and B, for determining the total iron.

Reagent A—To 1 gm. of protocatechuic acid in a 100 ml. volumetric flask add 50 per cent ethyl alcohol, dissolve at room temperature, and dilute to 100 ml. with the solvent. This reagent is kept in a dark glass-stoppered bottle away from sunlight.

Reagent B—To 1 part of N NH_4OH add 4 parts of N $(NH_4)_2SO_4$. All of the chemicals and solutions must be of the purest grade, as free from impurities as possible.

Both reagents remain unchanged for a long time if carefully handled.

Procedure—Pyrex test-tubes graduated at 1, 2.5, 5, 7.5, and 10 ml. are used in our technique. An exactly measured quantity of the solution to be tested is introduced into a test-tube, treated with Reagent A, and afterwards with Reagent B, drop by drop, with thorough mixing after each addition until the characteristic red color develops. The contents of the tube are then diluted to an appropriate volume. The solution must be clear and have a red color.

The photometric measurements are made with the Zeiss Pulfrich step-photometer. The technique employed in making the measurements is that described in the directions which accompany the instrument.[1] The extinction, E, was either read from a calibrated drum on the photometer or calculated from the transparency, D. The extinction coefficient, k, was then obtained by dividing E by s, the length of the cell used; $k = E/s$. A blank solution is used for reference liquid.

Effect of Hydrogen Ion Concentration on Color—The hydrogen ion concentration has a great effect not only on the consecutive colors culminating in the characteristic red, but also on their intensities. The blue-green color is not formed with protocatechuic acid in strongly acid solution of ferric ions; this color develops, changes to blue, and afterwards to violet, at about pH 5, in proportion to the diminution of the free H ions. These colors present various hues, and increase in intensity in proportion to the decrease of the hydrogen ion activity. The red develops in alkaline solution. The blue-green color disappears

altogether in strongly acid solution, and an excess of alkali hydroxide destroys the red color.

The influence of the hydrogen ion concentration on the various colors is shown in Table I. In this study the amount of iron was kept constant.

The determination of the *total iron* in acid solution, while practicable, is not advantageous, since it is essential to adhere to rigidly specified conditions, particularly with regard to the hydrogen ion concentration, and ferrous ion must be absent from the sample to be analyzed. In addition, the color reaction in acid solution of ferric ion is less sensitive. On the other hand, the protocatechuic acid reaction can be applied to the determination of both ferric and ferrous ions on the same solution by deter-

TABLE I

Effect of Hydrogen Ion Concentration on Color

pH	Color	Filter No.	k
2.85	Blue-green	S-66.6/3.5	0.156
4.00	"	S-66.6/3.5	0.201
4.54	Blue	S-61	0.284
5.00	Violet	S-57	0.357
5.10	"	S-57	0.357
5.80	"	S-57	0.482
8.50	Red	S-50	0.627

mining the ferric ion in acid solution and the total iron by the method described in this paper.

Effect of Excess of Reagents—An excess of Reagent B has no effect on the transparency. The same does not hold for Reagent A which carries a barely perceptible touch of yellow into the blank solution. This extremely slight coloring deepens in proportion to both the amount of protocatechuic acid and the time that the solution is allowed to stand before the extinction is measured, as shown by the following results. The final volume of the solution was 10 ml. in each case.

Reagent A ml.	k
0.2	0.000
0.3	0.000
1.0	0.003
2.0	0.006

After 30 minutes standing, the extinction coefficient for the solution containing 0.3 ml. of Reagent A was $k = 0.009$, and the extinction for the solution to which 2 ml. of the same reagent were added was correspondingly higher. From these data it is concluded that Reagent A must be used discreetly and that unnecessarily large amounts of protocatechuic acid are not to be added to the sample to be analyzed.

Quantity of Reagents—0.2 ml. of Reagent A for 5 ml. final volume is the maximum amount used in our technique, this being sufficient to develop the maximum color from a 4 mg. per 100 ml. solution of iron. Under the conditions we have used, a large number of analyses can be carried out simultaneously, particularly if the test-tubes are kept in the dark, since, then, the oxidation of the protocatechuic acid is retarded and the changes of the extinction due to the oxidation of this reagent are, over a certain period, without any effect on the results.

Stability of Color—Under the conditions we have used, the extinction remains unchanged for at least 1 hour, as shown in the following experiments.

A 0.5 mg. per 100 ml. solution of iron was treated with 0.1 ml. of Reagent A, and made alkaline with Reagent B. The values of k were as follows:

Immediately	30 min. later	60 min. later	90 min. later
0.538	0.538	0.538	0.550

Under the same conditions, but with 0.4 ml. of Reagent A, the following values were obtained.

Immediately	30 min. later	12 hrs. later
0.538	0.550	0.669

In these experiments pure water was used for compensating liquid.

A 0.25 mg. per 100 ml. solution of iron was treated with 0.2 ml. of Reagent A, made alkaline with Reagent B, and the color reading taken promptly thereafter. The extinction coefficient, $k = 0.268$, remained unchanged for at least 1 hour, either distilled water or blank solution having been used for compensating liquid. The following changes were recorded 18 hours later: against distilled water, $k = 0.347$; against the reagent blank

solution, $k = 0.168$. Such variations as these are due to the oxidation of the protocatechuic acid, a strongly reducing substance which is easily oxidizable, particularly in alkaline solution, developing a yellowish hue which increases in degree on standing.

Effect of Temperature on Color Readings—Under the conditions of our technique, it was found that anywhere in the range of 20–30° the color readings were identical; temperature is thus unimportant in this range which covers the usual room temperature.

Effect of Final Volume on Color Readings—A change of the final volume has no effect on the transparency, as is shown in Table II.

Lambert-Beer Law—The intensity of the red color formed with protocatechuic acid in solutions made alkaline with Reagent B is determined by the amount of iron present. It is this direct

TABLE II

Effect of Final Volume on Color

The solution contained 0.5 mg. per 100 ml. of Fe in each case.

Final volume	Cell length	E	k
ml.	cm.		
2.5	0.5	0.268	0.536
5.0	1.0		0.538
7.5	1.0		0.538
10.0	1.0		0.538

relationship between the iron and the intensity of color which permits the use of the method. The conformity of the colored solution to the Lambert-Beer law was tested. Calibration graphs plotted with the per cent of iron and extinction coefficient, k, as coordinates were straight lines; an equation for the per cent of iron may thus be derived from the slope of the line.

The extinction coefficient, k, is the extinction for a cm. layer of absorbing medium, and is directly proportional to the concentration of a solution, if the Lambert-Beer law holds. In this case, the concentration of the solution varies directly as the extinction coefficient, and to determine the concentration of an unknown we measure only the extinction of a layer of known thickness. We can then calculate the concentration of the solution.

Let k_1 denote the extinction coefficient of a solution of known concentration, c_1. The concentration of an unknown, c_2, giving an extinction coefficient k_2 is $c_2 = c_1/k_1 \times k_2$; k_2 and c_2 are variables and c_1/k_1 is a constant.

Once determined, the constant will always hold for the method; so that the concentration of an unknown is readily calculated by multiplying the constant by the extinction coefficient.

Table III shows that the extinction coefficient, k, is directly proportional to the concentration of iron, c.

TABLE III

Relation of Extinction Coefficient, k, to Concentration of Iron, c

Fe	Cell length	D	E	k	$\frac{c}{k}$
mg. per 100 ml.	*cm.*				
0.025	5.0	73	0.137	0.027	0.926
0.050	3.0	69	0.161	0.054	0.926
0.100	2.0	61	0.215	0.108	0.926
0.200	1.0	61		0.215	0.930
0.300	1.0	47.5		0.323	0.929
0.400	1.0	37		0.432	0.926
0.500	1.0	29		0.538	0.929
1.000	0.5	29	0.538	1.076	0.929
1.500	0.5	15.8	0.801	1.602	0.936
2.000	0.25	29.2	0.535	2.140	0.935
3.000	0.25	15.8	0.801	3.204	0.936
Mean...................	....	..	....		0.930

Standard error, $\sqrt{\Sigma(d^2)/(n(n-1))} = 0.0012$.

Interfering Ions—Interference by ions in colorimetric work is regarded as being caused by the color of the ion or by the fact that the ion forms a colored complex with the reagent. On the other hand, it is known that there is little correlation between the absence of color and the degree of interference, since an ion may form a colorless complex which is more stable than the desired colored complex.

The solution of iron to be tested must be colorless and clear, and neither color nor cloudiness should develop when a portion of the liquid is made alkaline with Reagent B alone. In view of the sensitivity of the protocatechuic acid reaction, very small

amounts of material are, as a rule, required for the determination of iron.

Copper interferes owing to the formation of the blue copper-ammonium complex. Since only small amounts of copper are ordinarily found in biological materials, the effect of this element with regard to the blue color formed by the interaction of copper and ammonium can be disregarded. The same holds for other ions which, like copper, may form colored complexes with Reagent B.

A number of types of biological material contain copper in the same order of magnitude as iron; however, the present method can be applied to the determination of iron in such materials without any modification. Under the conditions of our technique, a possible influence of copper on the oxidation of Reagent A in alkaline solution was not noted, and at concentrations up to 0.8 mg. of copper per 100 ml. no effect was recorded.

A number of ions interfere because they precipitate or cause a turbidity under the conditions of the color formation. For instance, the presence of large amounts of phosphate in biological material causes precipitation under the conditions of the method. Considering the quantity of ions generally found in such material, the phosphates to be taken into consideration are those of calcium and magnesium. The danger of precipitation of $MgNH_4PO_4$ is remote, since the development of the color is carried out at room temperature and is completed in several minutes. Calcium phosphate is formed at about pH 6.8 and may interfere seriously. Since an excess of citrate ion dissolves calcium phosphate, we preclude its formation by means of ammonium citrate.

Effect of Citrate Ion on Development of Color—We have studied the effect of citrate ion on pure solutions of iron, and on iron solutions to which calcium (as calcium chloride) and phosphorus (as ammonium phosphate) were added. No influence on the extinction was recorded, as may be seen in Table IV.

The maximum color develops promptly in pure solutions of iron, while the red color from iron solutions to which calcium, phosphorus, and ammonium citrate are added reaches full development only about 5 minutes after the reagents are added. This was shown by a solution containing 0.25 mg. of iron, 42.8 mg. of phosphorus, and 28 mg. of calcium per 100 ml., treated

with 1 ml. of a 20 per cent solution of ammonium citrate. The values of k were as follows: immediately 0.131, 3 minutes later 0.208, 8 minutes later 0.268, 20 minutes later 0.268.

Determination of Iron in Biological Materials

The determination of the total iron in any organic matter resolves itself into two main steps, ashing of the sample and determination of the iron in the ash.

Ashing of Sample—The greatest difficulty to be overcome is the possibility of loss of iron as the chloride. Methods of ashing have been designed to minimize this loss as much as possible, an object which is only approximated by many. After much study,

TABLE IV

Effect of Citrate, Calcium, and Phosphate Ion on Color

The solution contained citrate in each case.

Fe	Ca	P	k
mg. per 100 ml.	*mg. per 100 ml.*	*mg. per 100 ml.*	
0.50	0	0.0	0.538
0.50	28	0.0	0.538
0.50	0	42.8	0.538
0.25	28	42.8	0.268
0.50	14	21.4	0.538

the following wet ashing procedure (11) proved to be quite reliable and was finally adopted for general application.

A convenient sized sample of material is accurately measured and placed in a 200 × 20 mm. Pyrex test-tube graduated at 1, 2.5, 5, 7.5, and 10 ml. 1 ml. of 2 N H_2SO_4 is added and the test-tube is gently heated over a small flame, with continuous shaking, until acid fumes begin to form and the solution starts charring. Heating is stopped at this stage, and 1 ml. of a nitric-perchloric acid mixture (3:1) is added. The test-tube is gently heated until dense fumes of perchloric acid begin to form. Owing to the small amounts of material required for the determination of iron by our method, the solution is ordinarily colorless at this stage. Otherwise, further 0.5 ml. additions of the acid mixture are made, as the solution begins to char, until the oxidation has practically ceased. The test-tube is then heated more vigorously

until the perchloric acid has been completely eliminated and most of the sulfuric acid has evaporated. The resulting acidity is sufficient to secure easy dissolution of the ash, and to prevent iron oxide from being formed. 1 ml. of distilled water is added, and the test-tube is slightly heated and then cooled. Usually the solution is clear, but if a precipitate of calcium sulfate is present, as with samples of milk, for example, in which there is a large proportion of calcium to iron, this is filtered by suction on a fine Jena glass filter, and the filter is washed with distilled water. The solution may be concentrated, if necessary.

Determination of Iron in Ash—To the cold, acid solution of the ash 1 ml. of a 10 per cent solution of ammonium citrate, 0.05 to 0.2 ml. of Reagent A, and Reagent B, drop by drop, are successively added with thorough mixing after each addition until the characteristic red color develops. The contents of the tube are then diluted to an appropriate volume. A blank is carried through the entire procedure at the same time, under exactly the same conditions, to serve as compensating liquid. By so doing the effect of any iron contamination in the reagents is automatically eliminated.

The photometric measurements are made with the Zeiss Pulfrich photometer. The colored iron solution is run into the absorption cell of the instrument, the blank is placed in the other cell of the photometer, and the extinction is determined with the use of Filter S-50 and cells of appropriate length. Such a depth of cell is selected that the extinction, E, lies between 0.15 and 0.80; measurements of E above 1.00 and below 0.10 should be avoided as far as possible. One may work at extinctions below 0.10 and above 1.00, but the accuracy will suffer. The change of cells is not an inconvenience, and the proper size can be easily selected. All measurements are reduced to the common basis of a cell 1 cm. in length. The color readings must be made within 10 to 40 minutes after the addition of the reagents.

If too large a sample has been taken for the analysis, as sometimes may happen with material of unknown iron concentration, the final colored solution may be diluted to an appropriate volume before the color is read. If the solution of the ash should prove to be too concentrated, an aliquot of it may be diluted with distilled water to a suitable volume before the reagents are added.

Results

In order to determine the accuracy of the method, a sample of beans (*Phaseolus vulgaris*, L.) was analyzed by our method and also by the permanganate procedure. In Table V are presented the iron values obtained by our method. Determinations were

TABLE V

Representative Iron Analyses on Phaseolus vulgaris by Author's Method

Dry matter	Final volume	Cell length	D	E	k	Fe
mg.	*ml.*	*cm.*				*per cent.*
25.80	5	5	49.5	0.305	0.061	0.011
34.20	5	5	37	0.432	0.086	0.012
45.00	10	3	69	0.161	0.054	0.011
58.20	10	3	62	0.208	0.069	0.011
59.80	7.5	2	62	0.208	0.104	0.012
65.80	7.5	2	59	0.229	0.115	0.012
87.10	7.5	2	47	0.328	0.164	0.013
112.00	5	1	54		0.268	0.011
169.20	5	1	40		0.398	0.011
225.10	5	1	29		0.538	0.011

TABLE VI

Determination of Iron Content of Blood

Blood	Final volume	Cell length	D	E	k	Fe
ml.	*ml.*	*cm.*				*mg. per 100 ml.*
0.01	10	3	44	0.357	0.119	55.34
0.02	5	1	57.5		0.240	55.80
0.03	5	1	43.5		0.362	56.11
0.04	5	1	33		0.482	56.03
0.05	5	1	25		0.602	55.99
0.10	5	0.5	24.6	0.609	1.128	56.64

made on the same sample by the permanganate procedure. To cite only one determination taken at random, we have found 0.012 per cent of iron by employing 24.3174 gm. of the sample. As may be seen, the agreement between the two methods is quite satisfactory. The data given in Table V show the accuracy of our method compared with a reliable macromethod.

Determination of Iron in Blood—The determination of the amount of iron in the blood furnishes an accurate method for determining the hemoglobin content. Determinations can be made with very small amounts of blood by our method, and the results are quite reliable, as shown in Table VI.

SUMMARY

A new photometric method for the determination of iron with protocatechuic acid is described.

The various colors formed by the interaction of iron and protocatechuic acid have been studied, and the red color produced in alkaline solution has been selected for the determination of total iron, owing to the extreme sensitivity of this color reaction and to the fact that both ferric and ferrous ions behave identically under the conditions of the method.

The lack of interference, under the conditions of our method, by all ions commonly found in biological materials and the reproducibility and sensitivity of the color reaction make the method superior to other colorimetric procedures for iron.

The color system follows the Lambert-Beer law; therefore the concentration of an unknown is readily calculated. With the exception of a Pulfrich photometer, no special equipment is required, and, as no separations are involved, the procedure is very simple.

A simple, yet reliable, method of ashing the sample is described, and analyses for beans and blood are given.

The possibility of applying the protocatechuic acid reaction to the determination of both ferric and ferrous ions on the same solution is suggested.

Simplicity, rapidity, sensitivity, and applicability with minor variations to a wide variety of materials are the chief advantages of the protocatechuic acid method for iron.

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THE USE OF OPTICAL ROTATION IN THE STUDY OF PROTEIN HYDROLYSIS

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Change in optical rotation was first used as a measure of protein digestion by Schütz in 1885 (1). This investigator found that the amount of ovalbumin digested by pepsin, as measured by the optical rotatory power of the non-protein products, was proportional to the square root of the quantity of enzyme used. This relationship is commonly known as the Schütz or Schütz-Borissov rule. Schütz boiled the digestion mixture with ferric chloride to precipitate any protein residue, and found that the clear filtrate, which contained the hydrolytic products, was highly levorotatory.

Despite the simplicity and accuracy offered by optical rotation measurements, very little use has been made of this method for following protein hydrolysis. Gore (2) showed that when gelatin was digested by papain there was no longer a large change in specific rotation of the protein with temperature and that there was a regular relation between the activity of the enzyme used and the change in specific rotation of the substrate. Quick (3) utilized these facts to develop an empirical procedure for measuring the quantity of proteolytic enzyme present in various malts by the rate at which these enzymes decreased the specific rotation of gelatin solution.

The cleavage of synthetic peptides by trypsin and erepsin has been followed by change in optical rotation (4), but the accuracy was limited by the fact that, in most cases, the observed changes in rotation were rather small.

In the present study, optical rotation is shown to be a criterion of both the amounts and kinds of degradation products produced by either enzymic or acid hydrolysis of proteins. Only "com-

plete" proteins, precipitable by trichloroacetic acid, are used as substrates, so that, following enzymic digestion, measurements can be made exclusively upon the clear trichloroacetic acid filtrates, which contain only non-protein fractions, and not upon whole digestion mixtures.

Since total nitrogen analyses were always carried out on the non-protein fractions, the optical rotatory power of these solutions is expressed by an arbitrary term, which we define by analogy with specific rotation, as

$$\alpha N_D^{25} = \frac{100 \times \text{observed rotation, degrees}}{\text{length of tube, dm., } \times \text{ gm. total N per 100 ml.}}$$

αN_D^{25} can be converted to ordinary specific rotation, α_D^{25} (or *vice versa*), by multiplying the former quantity (or dividing the latter) by the fractional percentage of N which the optically active substances in solution contain. The method of obtaining this percentage will be indicated subsequently for specific cases.

When the hydrolysis is with mineral acid, samples of the protein hydrolysate are decolorized with carbon and filtered, and analyzed in the same manner as the above trichloroacetic acid filtrates.

It will be shown that αN_D^{25} is related to the percentage of free amino groups in the degradation products of casein, so that the former quantity is a measure of the degree of splitting of the peptide bonds. This fact is utilized in the comparison of the action of different proteases on the same protein substrate.

An indication that enzymic hydrolysis proceeds by a somewhat different path than that followed during acid hydrolysis is obtained by correlating the changes in αN_D^{25} of the different non-protein fractions with corresponding changes in the ratio of free amino N to total N, —NH₂:N.

<center>EXPERIMENTAL</center>

Proteolytic Enzymes—The preparation of the plant proteases, papain, bromelin, asclepain, and solanain, in partially purified form has been described by us (5, 6). The solutions of these enzymes used in subsequent measurements contained 5 mg. of enzyme per ml. of 0.05 M NaCN, adjusted to pH 7. The animal proteases used were Armour's trypsin and Merck's pancreatin. These preparations are, of course, crude mixtures of enzymes, but

were quite satisfactory for the present purpose. Filtered, aqueous extracts were used for the digestions.

Sheep erepsin was salted-out of an aqueous extract of ground duodenal tissue by two-thirds saturation with solid $(NH_4)_2SO_4$. The precipitate was dialyzed, and an aqueous extract of this material was used. The hog erepsin[1] used in certain experiments was a 30 per cent glycerol extract of hog intestinal mucosa, and was further activated by the addition of dilute Mn^{++}. The yeast polypeptidase was a concentrate prepared by Dr. M. J. Johnson.[1] It was further activated by dilute Zn^{++}.

Proteins—The proteins used were ovalbumin, prepared by Sörensen's method (7), twice recrystallized, and then dialyzed; Van Slyke casein (8); and edestin prepared according to Osborne (9).

Methods

For the enzymic digestions, 2 ml. portions of enzyme solution were added to 15 ml. volumes of protein solution, buffered at pH 7.5 to 8.0. After a specified time of digestion, usually at 37°, 5 ml. of 20 per cent trichloroacetic acid solution were added to each digest. The mixtures were filtered, and their optical rotations were measured in a 2 dm. polarimeter tube with a Schmidt and Haensch polarimeter. A General Electric sodium vapor lamp was the source of illumination. The temperature of the measurements was 25°, except when otherwise specified. Several readings were usually made on each solution, and the average value taken.

Following the rotation measurements, the same solutions were analyzed for total N by the micro-Kjeldahl method and for amino N by the micromethod of Van Slyke. In the latter method, the HNO_2 was allowed to react for 5 minutes at about 20°.

In some instances the tyrosine color value was also measured, according to Anson's method (10). In these latter measurements the intensity of the blue color produced with the phenol reagent was determined with an Evelyn photoelectric colorimeter. This

instrument was calibrated against a series of standard tyrosine solutions.

In certain cases in which the substrate was subjected to the action of two or three different enzyme preparations, the digestion mixture was briefly heated at 95–100° to inactivate each enzyme prior to the addition of the next one.

In all cases, control measurements were carried out in which the trichloroacetic acid solution was first added to the protein substrates and the enzyme solutions added afterwards. These corrections, which were subtracted from the experimental values, usually amounted to from 0° to 0.10° of optical rotation per 2 dm., 0.1 to 0.2 mg. of total N per ml., 0.05 to 0.1 mg. of amino N per ml., and 0 to 0.005 × 10⁻² milliequivalent of tyrosine color per ml. for the digestions with the plant proteases. The corrections were about twice as large when pancreatin, trypsin, or the peptidase preparations were used.

The optical rotation readings made on each solution usually agreed to within 0.02° to 0.05°, so that, for an observed rotation of 2° to 5°, the relative error was about 1 per cent.

Effect of Varying Temperature and pH on Optical Rotation of Non-Protein Fraction—Measurements at different temperatures were made on the trichloroacetic acid filtrate from a papain digest of casein, a polarimeter tube with an outer metal jacket for water circulation and temperature control being used for this purpose. It was found that, between 16.6–37.5°, the greatest range in the observed rotations was from −3.04° to −3.12°, a variation corresponding to approximately twice the limit of the experimental error. Accordingly, it was not necessary to specify accurately the temperature at which all subsequently recorded measurements were made, and 25° (the usual temperature of the polarimeter room) is taken as the standard temperature.

In order to determine the effect of varying pH on optical rotation, 10 ml. portions of the trichloroacetic acid filtrate of a tryptic digest of casein were adjusted to different pH values with acid or alkali and made up to a 25 ml. volume with water. Between pH 1 and 7, the observed rotations increased gradually from −3.56° to −3.87° and then gradually fell to −3.76° at pH 10. In view of the great variation of rotation with pH in the case of many proteins and of all amino acids (11), this relative constancy in the

rotation values of the tryptic digestion products is surprising, and cannot be explained by us. When the final concentration of trichloroacetic acid was varied between 2 and 7.5 per cent, there was no appreciable effect produced on the optical rotation.

Optical Rotation Measurements Following Enzymic Digestion in Urea Solution—The digestion of most denatured proteins in concentrated urea solutions can be followed by optical rotation changes as readily as in the case of aqueous solutions, except that (owing to the presence of urea) amino and Kjeldahl N determinations cannot be made on the filtrates. Hemoglobin is not a suitable protein for these measurements, since, upon prolonged digestion, some of the red color due to heme is carried over into the trichloroacetic acid filtrate, and interferes with the optical rotation readings.

Concentrated urea is known to increase the specific rotation of ovalbumin (12). This was likewise observed to be true for casein and its intermediate digestion products. Thus we found $\alpha_D^{25} = -144°$ for casein in 6.6 M urea at pH 7.5. This figure is about 45 per cent higher than the value $\alpha_D^{25} = -100°$, reported by Almquist and Greenberg (13) for aqueous casein solutions at the same pH.

As an example of the measurement of the rotation of proteolytic digestion products in urea, 12 ml. of 3.12 per cent casein in 6.6 M urea[2] were digested by 2 ml. of papain solution for 20 hours at 37°. 5 ml. of 20 per cent trichloroacetic acid were then added, and the resulting very slight precipitate filtered out. The filtrate, which contained all of the original casein N, and which was in 4 M urea, gave a value of $\alpha N_D^{25} = -655°$. This value is about 10 per cent higher than that subsequently reported for aqueous casein digests.

Comparison of Optical Rotation with Other Methods for Measuring Rate of Enzymic Proteolysis—Fig. 1 illustrates the use of four independent methods for measuring the rate of digestion of casein by papain. The curves are all very much alike and all level out after about 2 hours. This is the time which was required for the complete conversion of the original casein to the non-protein form. This latter fact was ascertained by comparing the concentration of total N in the trichloroacetic acid filtrates with that in the ini-

[2] The percentage of casein in the substrate was calculated from the total N content, determined prior to the addition of urea.

tial substrate, after correction for the dilution of the filtrates. Also, after about 2 hours, there was no appreciable precipitate when the trichloroacetic acid was added to the digestion mixture.

If the final tyrosine color value of 0.90×10^{-2} milliequivalent per ml. is used to calculate the percentage of tyrosine in the original casein, a value of 7.1 per cent is obtained upon correction for the dilution with enzyme and trichloroacetic acid solutions
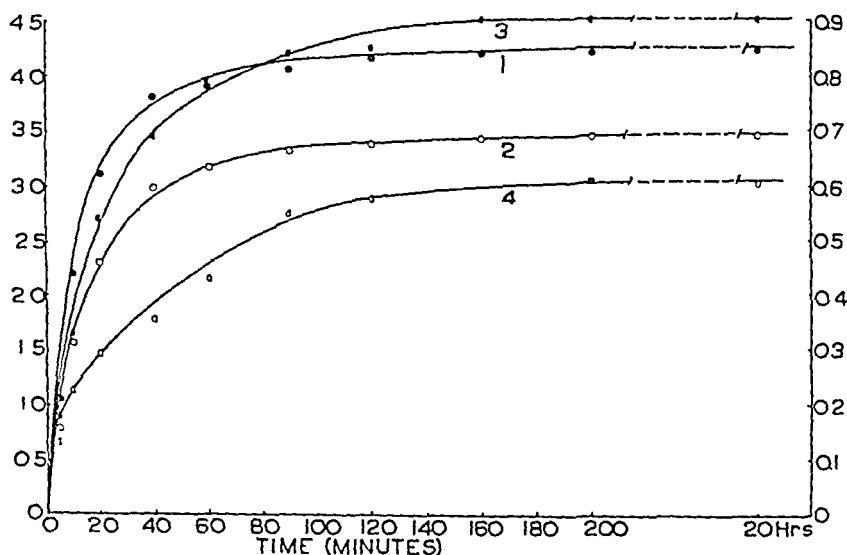
FIG. 1. Comparison of the optical rotation with other methods for meas uring the rate of formation of non-protein fractions in the digestion o casein with papain. 15 ml. portions of 3.4 per cent casein were digested at 40° for different times by 2 ml. of papain solution. Scales for the curves are represented on the ordinates as follows: Left ordinate, Curve 1, optical rotation in levo degrees per 2 dm.; Curve 2, total N in mg. per ml. Right ordinate, Curve 3, tyrosine color value in milliequivalents $\times 10^2$ per ml.; Curve 4, amino N in mg. per ml.

This is in fair agreement with the value of 6.55 per cent found by Folin and Ciocalteau (14), considering that the former figure is uncorrected for tryptophane, which is present to about 2 per cent but which gives a much less intense color with the phenol reagent. All other proteases which were tested on casein produced essentially the same final tyrosine color value in the digestion products; namely, that corresponding to 6.8 to 7.5 per cent tyrosine (plus tryptophane) in the original casein.

Correlation of αN_D^{25} and $-NH_2:N$ with Time and Degree of Hydrolysis—Curves 1 and 2 of Fig. 2 show that, when casein is hydrolyzed by papain at 40°, the values of αN_D^{25} and $-NH_2:N$ in the non-protein filtrate do not change greatly throughout the whole time of the digestion. The final value, $\alpha N_D^{25} = -600°$, of the digestion products is only about 15 per cent lower than that of the original casein, $\alpha N_D^{25} = -710°$.[3] Digestion with papain at a higher temperature, 60°, yielded essentially the same results
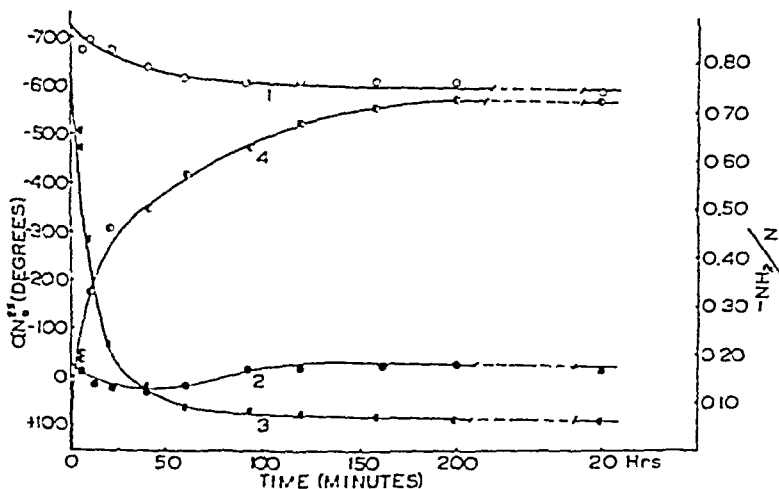
FIG. 2. αN_D^{25} and $-NH_2:N$ as functions of the time of hydrolysis of casein. Hydrolysis with papain, Curve 1, αN_D^{25} *versus* time in minutes; Curve 2, $-NH_2:N$ *versus* time in minutes. These curves were calculated from the data of Curves 1, 2, and 4 of Fig. 1. Hydrolysis with HCl, Curve 3, αN_D^{25} *versus* time in minutes ÷ 10; Curve 4, $-NH_2:N$ *versus* time in minutes ÷ 10. These curves were obtained by hydrolyzing 12 gm. of casein with 300 ml. of 1:5 HCl, and analyzing aliquots, removed after different times, for optical rotation, amino N, and total N.

The final value, $-NH_2:N = 0.175$ (Curve 2), compared to the ratio 0.73, obtained by acid hydrolysis[4] (Curve 4), indicates that about 24 per cent of all the peptide bonds are split by the papain.

[3] This value, determined at pH 3.0, corresponds to $\alpha_D^{25} = -111°$, assuming 15.6 per cent N in the casein.

[4] This value is about equal to the ratio 0.724 found by Hunter and Smith (15), and slightly higher than the ratio 0.70 reported by Dunn (16). Our amino N values may include a small percentage of ammonia N, since the

It is noteworthy that αN_D^{25} and $-NH_2:N$ (for enzymic hydrolysis) become constant after about 2 hours. As previously mentioned, this is also the time required for all of the original casein to be converted to the non-protein form. Evidently longer times of digestion with the papain caused no further degradation of the non-protein substances.

With acid hydrolysis,[5] the rotation is seen to change from the initial value for casein, $\alpha N_D^{25} = -710°$, to $\alpha N_D^{25} = +88°$ for the final hydrolysate; i.e., the amino acid mixture, after about 2000 minutes or 33 hours. With 13.7 per cent as the N content of this mixture (on a dry basis),[6] $\alpha N_D^{25} = +88°$ corresponds to $\alpha_D^{25} = +12.0°$. It is interesting that this last value agrees surprisingly well with the value, $\alpha_D^{20} = +10.8°$, for the complete amino acid mixture (excluding tryptophane), calculated solely from a consideration of the optical form of the individual amino acids of casein (19), their specific rotations in HCl solution (11), and the percentage to which each is present in the protein (18). This agreement suggests that there is no extensive racemization of the amino acids during the hydrolysis of a protein with dilute acid.

Comparison of Paths of Acid and Enzymic Hydrolysis of Casein— The comparison is shown in Fig. 3. Curve A, which represents acid hydrolysis, is obtained from Curves 3 and 4 of Fig. 2 by

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ammonia due to amide N was not removed from the trichloroacetic acid filtrates. About 10 per cent of the total N of casein is amide N (16), and Van Slyke (17) has shown that a third of the NH_3 of 0.1 N $(NH_4)_2SO_4$ reacts with HNO_2 in 5 minutes at 24°. This last fact was confirmed by us. We did not determine whether papain liberates NH_3 from casein, but it appears likely that this occurs, since tryptic hydrolysis liberates a fourth of the amide N of casein (15), and papain is known to split hippurylamide.

[5] After the first 15 minutes of boiling with HCl, the casein hydrolysate gives only a very slight precipitate with trichloroacetic acid, and after an hour none at all. This indicates that the protein is very rapidly degraded to the non-protein stage.

[6] From the amino acid composition of casein (18), the average weight of the amino acid residues was calculated to be 123. Tryptophane, which is destroyed by acid hydrolysis, is not included in this calculation. Assuming the addition of 18 gm. of water during complete hydrolysis, the N content drops from 15.6 to $123/141 \times 15.6 = 13.6$ per cent. Essentially the same value, 13.8 per cent, was calculated by considering the percentage of N in each amino acid and the percentage to which each (excluding tryptophane) is present in the casein.

plotting the αN_D^{25} values against the corresponding values of the —NH$_2$:N ratios.
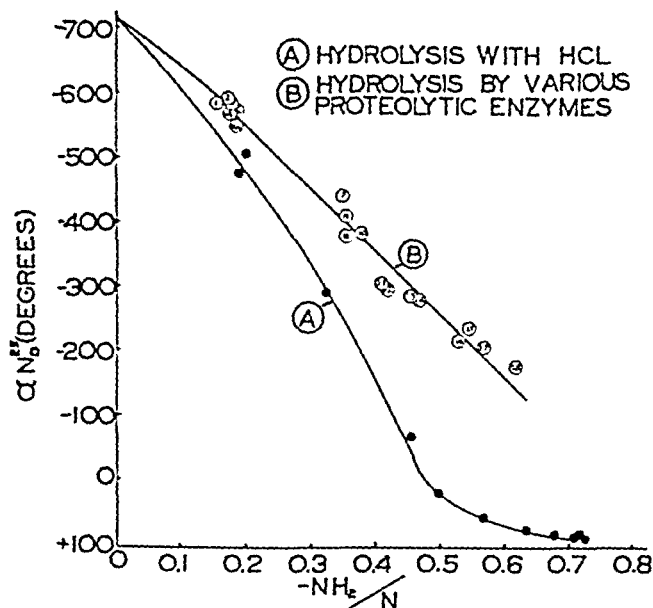
FIG. 3. αN_D^{25} as a function of —NH$_2$:N in acid and in enzymic hydrolysis of casein. The points of Curve B represent digestions of 15 ml. portions of 4.0 per cent casein at 37° by 2 ml. portions of different enzyme solutions, in the order, and for the time specified below: (1) solanain, 20 hours; (2) papain, 20 hours; (3) bromelin, 20 hours; (4) asclepain, 20 hours; (5) papain and bromelin (acting simultaneously), 20 hours; (6) papain + solanain (acting simultaneously), 20 hours; (7) pancreatin, 18 hours; (8) papain, 18 hours, + pancreatin, 20 hours; (9) papain, 18 hours, + trypsin, 20 hours; (10) trypsin, 18 hours; (11) trypsin, 20 hours, + papain, 20 hours; (12) trypsin, 20 hours, + hog erepsin, 3 hours; (13) trypsin, 20 hours, + sheep erepsin, 18 hours; (14) trypsin, 20 hours, + hog erepsin, 9 hours; (15) trypsin, 20 hours, + yeast polypeptidase, 3 hours; (16) trypsin, 20 hours, + yeast polypeptidase, 26 hours; (17) trypsin, 20 hours, + hog erepsin, 9 hours, + yeast polypeptidase, 17 hours; (18) trypsin, 20 hours, + yeast polypeptidase, 26 hours, + papain, 2 hours (at 60°). 0.5 ml. volumes of yeast polypeptidase solution (instead of 2 ml.) were used for the digestions.

Curve B is the composite result of a number of separate digestion experiments made with different enzymes and enzyme combinations on the same casein substrate. The conversion of casein

to non-protein material was 95 to 100 per cent complete in all these cases. The papainases, papain, bromelin, and asclepain, are seen to yield almost identical values (points *2* to *4* on Curve B), so that their mechanisms of action on casein are very likely the same. In agreement with this view is the observation that the combination of papain and bromelin (point *5*) has no different effect than has either protease acting alone.

The non-protein product produced by solanain (point *1*) appears to have approximately the same αN_D^{25} value, $-580°$, as in digestions with the above papainases, but the ratio $-NH_2:N = 0.155$ is slightly lower, indicating that this enzyme split only about 20 per cent of the peptide bonds of casein, whereas the papainases split about 25 per cent of the bonds. It is interesting that the behavior of solanain in the present experiments is so similar to that of the papainases, since it is not a member of this group of proteases (6). Point *6* shows that solanain is not able to further the degree of hydrolysis when acting in conjunction with papain.

Pancreatin (point *7*) and crude trypsin (point *10*) yield products which have lower αN_D^{25} values, and $-NH_2:N$ ratios of about 0.35 to 0.38. These last values indicate that approximately 50 per cent of the peptide bonds of casein are broken, and compare very well with the value of 0.374, reported by Hunter and Smith (15), for a 24 hour digestion of casein with crude trypsin. These workers found the initial rate of splitting to be very rapid, but were able to obtain much higher amino N values by prolonging the digestion over many days. For example, Dauphinee and Hunter (20) obtained the ratio $-NH_2:N = 0.466$ for a 14 day digestion of casein with trypsin. In our experiments, digestions with different enzymes were not prolonged for more than a single day, and the amount of enzyme used was small in relation to the quantity of substrate.

Points *8* and *9* suggest that when papain acts on casein prior to the pancreatic proteases, the net hydrolysis is the same as when the latter enzymes act alone on casein. However, point *11* indicates that when papain acts on the tryptic digest, there is a certain amount of further hydrolysis.

Points *12* to *14* indicate the very similar action of sheep and hog crepsin on tryptic digests of casein. These peptidase mixtures,

like the plant proteases, complete their action in a short time, and yield no further hydrolysis over longer periods of time.

Yeast polypeptidase, acting subsequent to trypsin (points *15* and *16*), and in combination with erepsin (point *17*) and papain (point *18*), yields a still higher degree of hydrolysis.

In view of the fact that optical rotation is a function of molecular structure, the spread between Curves A and B suggests that the proteolytic hydrolysis of casein proceeds through a somewhat different set of intermediate substances than with acid hydrolysis. It is significant that the solution corresponding to point *18* of Curve B gave a positive (pink) biuret test, although the ratio $-NH_2:N = 0.62$ indicates that about 85 per cent of all the original peptide N was in the free amino form. Apparently some tripeptides, and probably even higher polypeptides, were still present in the solution. However, a point on Curve A having approximately the same $-NH_2:N$ ratio, 0.635, but a very different αN_D^{25} gave a negative biuret reaction. This indicates that there were no molecules larger than dipeptides in the latter solution.

These observations confirm the above view that the enzymic and acid hydrolysis of casein follows different paths. Presumably Curve B should meet the lower tip of Curve A if the same final products (simple amino acids only) are obtained by either method.

Application of Optical Rotation Method to Ovalbumin and Edestin —The results in Table I show that ovalbumin and edestin resemble casein in that the initial αN_D^{25} and $-NH_2:N$ values of the non-protein products are not greatly modified by prolonged digestion with papain.

It is interesting that the digestion products of ovalbumin have higher levorotation values ($\alpha N_D^{25} = -415°$ to $-360°$) than the original native protein. Almquist and Greenberg (21) give $\alpha_D^{22} = -35.1°$ for native ovalbumin at pH 1.72. This rotation corresponds to $\alpha N_D^{22} = -225°$, assuming 15.6 per cent N in ovalbumin. The higher rotation of the digestion products is probably related to the fact that the optical rotation of ovalbumin is greatly increased by denaturation (12, 22).

Osborne (23) gives the value $\alpha_D^{20} = -41.3°$ for native edestin in neutral 10 per cent NaCl solution. Since this protein has 18.8

per cent N, $\alpha N_D^{20} = -220°$. This last value is considerably smaller than those reported for the digestion products in Table I.

Calvery (24) gives 0.728 as the value of $-NH_2:N$ obtained by the complete acid hydrolysis of ovalbumin. Based on this figure, the value 0.175 reported in Table I for ovalbumin indicates that about 24 per cent of the peptide links were split by papain. This former value is much lower than the ratio $-NH_2:N = 0.51$ reported by Calvery for the digestion of ovalbumin by papain for a period of days. Calvery found that papain split two-thirds of the peptide bonds of ovalbumin, while pepsin and trypsin each split only a third of the bonds. He was able to obtain com-

TABLE I

Digestion of Ovalbumin and Edestin by Papain

Protein	Time of digestion	αN_D^{25}	$-NH_2:N$
	hrs.	*degrees*	
Ovalbumin	1	-415	0.15
	18	-360	0.175
Edestin	$\frac{1}{2}$	-325	0.11
	1	-320	0.115
	12	-300	0.125

15 ml. portions of 5 per cent solutions of ovalbumin and edestin, partially denatured by brief heating at 100°, were digested by 2 ml. of papain solution at 37°. The edestin solution contained 10 per cent NaCl to assist in keeping the protein in solution.

plete hydrolysis by using peptidases in conjunction with the above enzymes.

The ratio $-NH_2:N = 0.125$ reported in Table I for edestin is comparable to the value 0.147 reported by Dauphinee and Hunter (20) for an 8 hour digestion of edestin with crude trypsin. These workers obtained the ratio $-NH_2:N = 0.531$ when this digestion was prolonged for 35 days. The value 0.125 indicates a splitting of 20 per cent of the peptide bonds of edestin, the ratio 0.628 being taken to indicate the complete hydrolysis (20).

It is interesting that the $-NH_2:N$ ratios, resulting from the action of papain, are almost the same for the three different proteins, casein, ovalbumin, and edestin. This fact suggests that

papain hydrolyzes certain proteins to an approximately equal degree.

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SUMMARY

1. A quantitative method is described for measuring the amount of proteolytic digestion by determining the degree of optical rotation of the non-protein products. Casein, ovalbumin, and edestin are used to illustrate the method.

2. Changes in optical rotatory power are used in conjunction with the increase in free amino groups to follow the course of the acid hydrolysis of casein. The final value of the specific rotation of the hydrolysate agrees closely with· the theoretical value calculated from data given in the literature for· the different amino acids of casein.

3. By measuring the optical rotation and percentage of free amino nitrogen of the products resulting from the action of different plant and animal proteases and peptidases on casein, evidence is obtained which indicates that the enzymic and acid hydrolysis of this protein proceeds by somewhat different paths.

4. Evidence from optical rotation and amino nitrogen measurement with different plant enzymes suggests that these proteases hydrolyze casein in a very similar manner and yield the same non-protein products.

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ON THE PROPERTIES OF 2-METHYLTHIAZOLINE AND THEIR RELATION TO THE PROTEIN PROBLEM

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The problem of protein denaturation is still very obscure, particularly since we are unable to state clearly what we understand by denaturation. Different chemical changes in the protein molecule may probably lead to what with our present knowledge we call the same physical change, and, while exploring the chemical mechanism of the denaturation by one agent, we may find but few facts which will stand the process of generalization.

One chemical change following or causing denaturation seems to be general, though; *viz.*, the "appearance" of thiol groups (Harris (1)), (see also 2–5). Whichever agent is used, heat, a specific agent like guanidine salt, or a non-specific one like alcohol, the same effect is qualitatively obtained. Quantitatively, however, important differences have been demonstrated (6–8) indicating differences in the mechanisms of these processes.

Two views have been expressed concerning this appearance of thiol groups. One ascribes it to a simple liberation of these groups from chemical combinations into which they have entered with loss of their specific properties; *i.e.*, to a disruption of chemical bonds between the sulfur atom and other atoms. The other view maintains that the loss of reactivity of the thiol groups in the protein molecule may be due to steric hindrance, to a "concealment" of these groups in the interior of a vast structural network. This assumption implies that any chemical reaction which results in a partial or total degradation of the protein molecule may be involved in the process of denaturation.

It is not the purpose of the present paper to give a general discussion of this problem. We may point out that till now it has remained entirely unsolved, so that one point of view may be as

good as the other as a starting point for an investigation. The fact, however, that if we accept the first view there is more hope of a rapid solution by means of simple models makes it rather tempting to take up the study of sulfur compounds on this basis.

The following considerations led the authors to investigate the properties of a simple compound like 2-methylthiazoline.

Several years ago Bergmann and Miekeley carried out some important investigations on the behavior of oxazolines in aqueous solutions (9, 10). A compound like

$$\begin{array}{l} CH_2-O \\ \quad\quad\quad\quad >CC_6H_5 \\ CH-N \\ \quad | \\ COOH \end{array}$$
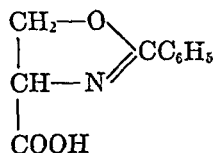
was found to undergo a rapid hydrolysis in acid solution, the chief product formed being O-benzoylserine. With increasing alkalinity, however, this O-benzoyl compound would tend to rearrange to N-benzoylserine with liberation of hydroxyl groups. Bergmann himself (10) has pointed out that such unstable oxazoline rings or rings of similar type may be present in proteins.

Considering now a compound like 2-methylthiazoline (A) (Gabriel and von Hirsch (11)), it is immediately seen that hy-

$$\begin{array}{l} CH_2-S \\ \quad\quad\quad\quad >CCH_3 \\ CH_2-N \end{array} \qquad\qquad \begin{array}{l} CH_2-SH \\ \quad | \\ CH_2-NHCOCH_3 \end{array}$$
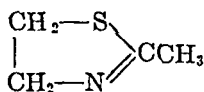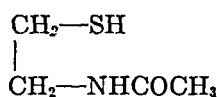
(A)   (B)

drolysis under suitable conditions might lead to a formation of N-acetylaminoethylmercaptan (B) and a liberation of a thiol group. In view of the possibility previously mentioned (12) that peptide bonds are set free in the process of denaturation, it is of interest to note that a peptide bond is formed at the same time

The properties of methylthiazoline were therefore subjected to a preliminary investigation with special regard to the stability of this compound under conditions resembling those at which denaturation of proteins is known to occur.

2-Methylthiazoline is a weak base, the pH at half ionization being 5.37 (in 0.1 M KCl at 25°). It is fairly stable in aqueous solution around neutrality but becomes increasingly unstable

with increasing acidity. The hydrolysis is a first order reaction (measured at 60° in buffers of different pH), and by plotting the reaction constants against pH a curve is obtained which coincides rather well with the ionization curve (Fig. 1). This shows that the methylthiazoline ion is the unstable molecule. The method for following the hydrolysis was based upon the reaction of porphyrindin with free thiol groups (13, 14, 7), and since the unhydrolyzed methylthiazoline does not react at all with this dye, while the theoretical amount of thiol groups is found after mild exhaustive hydrolysis (3 hours at pH 3 and 60°), we may conclude that whatever compounds are formed by the hydrolysis must contain a free thiol group or at least be capable of rearranging to
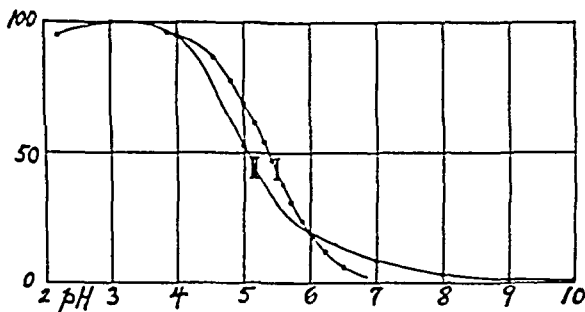
FIG. 1. Curve I, ionization curve of thiazoline; Curve II, hydrolysis curve of thiazoline. The ordinate scale shows the per cent ionization or hydrolysis respectively; rate at pH 3 = 100.

substances with free thiol groups under the conditions chosen for the thiol determination (20°, pH 6 to 7).

N-Acetylaminoethylmercaptan has no basic properties. Hence, by titrating the hydrolysates to pH 3, it is possible to distinguish between the formation of this compound and that of the basic S-acetyl compound. If the N-acetyl compound is formed exclusively, then 1 equivalent of base will disappear per molecule of thiazoline split. The formation of the S-acetyl compound, however, will be followed by no change in amount of base.

A preliminary investigation showed that the formation of N-acetylaminoethylmercaptan predominates within the pH range investigated, but that some S-acetyl compound is formed too,

presumably together with its split-products, acetic acid and aminoethylmercaptan.

We have not gone fully into this matter because the displacement of the acetyl group from —SH to NH_2 is rapid and complete in all cases in which the N-acetyl compound is removed in the process of analysis (oxidation, reaction with iodoacetate, etc.). Hence the chief result, that —SH groups "appear" when thiazoline is heated, is uninfluenced by the relative uncertainty concerning the split-products formed.

A substance such as diacetylcystine dimethyl ester, which may be regarded as representative of some sulfur-containing structures known to occur in proteins, behaves quite differently when heated to 60° at different pH values. The liberation of thiol groups, determined by oxidation with porphyrindin, proceeds extremely slowly at pH values below 7. Above pH 7, however, considerable amounts of thiol groups are set free immediately after the temperature is raised to 60°, while but a slight increase in this amount is observed after prolonged heating. The processes taking place seem to be extremely complicated.

It is not without interest to compare these results with results obtained by treating ovalbumin and insulin in a similar way. Ovalbumin was chosen as a representative of proteins with labile sulfur groups and with a pronounced tendency to denature, while insulin, which probably contains all its sulfur as cystine, was taken as an example of proteins with little or no tendency to denature.

Fig. 2 shows the curve found for ovalbumin. It will be seen that the sulfur groups in ovalbumin are distinctly acid-labile as in the case of thiazoline (compare (15, 16)). No thiol groups appear at pH 6 and 7 (even after prolonged heating) and at pH 8 to 10 the increase in reducing groups is but slight and may be explained as a destruction of S—S linkages in the protein. In 0.5 N NaOH this destruction was found to be very rapid, an abundant amount of reducing groups being formed. Ovalbumin differs here from thiazoline, which is but slightly hydrolyzed in 1 N NaOH.

Insulin, as distinct from ovalbumin, yields no measurable quantities of thiol groups in the whole acid region. A very slight formation is observed on the alkaline side of pH 7 except at pH 10 where a strong reaction seems to set in after a long induction period.

On the basis of these experiments, therefore, it would seem worth while to search for other similarities, and we started out by investigating the influence of specific denaturation agents like urea and guanidine salts on 2-methylthiazoline (8). Urea had no effect at all, but in solutions of guanidine chloride and bromide a distinct increase in thiol groups was observed. The effect was by far the largest in guanidine bromide solutions (compare Greenstein (8)) and we thought for a time that we had come across another and more striking similarity than that in the heat "denaturation."[1] We very soon found out, however, that the effect was due to an impurity in the salts applied and that the impurity was ammonium ions.
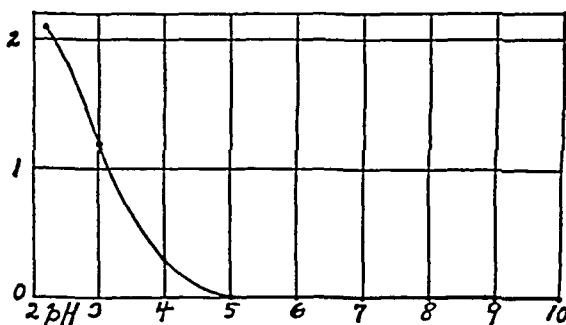
Fig. 2. Denaturation curve for ovalbumin. The ordinate scale represents the number of thiol groups per molecule of protein in 5 minutes at 60°.

In aqueous solutions ammonium ions react with methylthiazoline in a peculiar way. The reaction may be written

$$
\begin{array}{c}
CH_2—S \\
| \qquad\quad \diagdown CCH_3 + NH_4^+ = \\
CH_2—N \diagup
\end{array}
\qquad
\begin{array}{c}
CH_2—SH \quad NH_3^+ \\
| \qquad\qquad | \\
CH_2—N = CCH_3
\end{array}
$$

(x)

or

$$
\begin{array}{c}
CH_2—S \\
| \qquad\quad \diagdown CCH_3 + NH_4^+ = \\
CH_2—N \diagup
\end{array}
\qquad
\begin{array}{c}
CH_2—S—NH \\
| \qquad\qquad | \\
CH_2—N—CCH_3 \\
\qquad H_2^+
\end{array}
$$

(y)
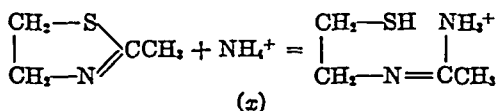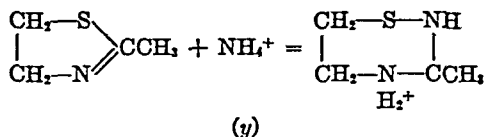
[1] We actually went so far that we sent a preliminary note to *Nature*. It was almost immediately withdrawn, however, by telegram and letter. We mention this because, owing to the European war, we have been unable to ascertain whether our messages were received.

In the latter case we shall have to assume that the hypothetical compound formed is in equilibrium with (x) or has reducing properties in itself, so that it may react with porphyrindin to form (z) which was definitely proved to be the final product after

$$\begin{array}{ccc}
CH_2 \text{---} S \text{---} S \text{---} CH_2 \\
| \qquad\qquad\qquad | \\
CH_2 \text{---} N \qquad N \text{---} CH_2 \\
\| \qquad\qquad \| \\
CCH_3 \qquad CCH_3 \\
| \qquad\qquad | \\
NH_3^+ \qquad NH_3^+
\end{array}$$

(z)
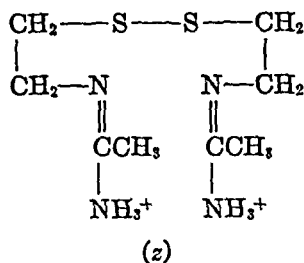
oxidation. The reduced thiazoline-ammonium ion compound was very difficult to isolate because of its instability. All our attempts resulted only in crystals of ammonium chloride imbedded in a viscous oil which smelled strongly of thiazoline and turned into a yellow paste on drying *in vacuo* over P_2O_5. We are therefore unable to distinguish between formulas (x) and (y) but for the time being we have chosen formula (x) as the simpler of the two.

The oxidized compound was isolated as follows:

1 ml. of thiazoline (10.8 mM) was mixed with 2 ml. of 5 N ammonium chloride solution (10.0 mM of NH_4^+), a trace of $FeSO_4$ was added, and the mixture shaken with air in a 600 ml. flask. The solution which at the beginning was distinctly reddish brown became colorless in the course of 24 hours, though with fine particles of ferric hydroxide. After 24 hours as much water as possible was distilled off *in vacuo* at 30° and alcohol and acetone added, whereupon a substance of the composition $C_8H_{20}S_2N_4Cl_2$ crystallized out in colorless needles. Yield, 1.50 gm., or almost theoretical; m.p. 177.5° (corrected); thiol reaction negative. N 18.13 (calculated 18.23), Cl 23.16 (calculated 23.08). Picrate, m.p. 184.0° (corrected).

180 mg. of the substance were dissolved in hot absolute alcohol and the solution cooled. The crystals formed were separated from the mother liquor and dried *in vacuo* over P_2O_5. Yield, 54.2 mg.; N 18.26 (calculated 18.23).

The mother liquor was precipitated with acetone. The new batch of crystals was dried *in vacuo* over P_2O_5. Yield, 84.2 mg.;

N 18.19, Cl 23.20 (calculated 23.08).   This shows that the substance is homogeneous.

The position of the ammonium ion taken up is determined in the following way.   We may consider two possibilities.

$$2 \; \begin{array}{c} CH_2-S \\ | \qquad\quad \diagdown \\ CH_2-N \diagup \end{array} CCH_3 + 2NH_4^+ \rightarrow 2 \; \begin{array}{c} CH_2NH_3^+ \quad \overset{H}{\underset{}{S}} \\ | \qquad\qquad | \\ CH_2-N=CCH_3 \end{array}$$

$$+ \tfrac{1}{2}O_2 \rightarrow 2 \left[\begin{array}{c} \qquad\qquad | \\ CH_2NH_3^+ \quad S \\ | \qquad\qquad | \\ CH_2-N=CCH_3 \end{array} \right]$$

(I)

$$2 \; \begin{array}{c} CH_2-S \\ | \qquad\quad \diagdown \\ CH_2-N \diagup \end{array} CCH_3 + 2NH_4^+ \rightarrow 2 \; \begin{array}{c} CH_2SH \quad NH_3^+ \\ | \qquad\qquad | \\ CH_2-N=CCH_3 \end{array}$$

$$+ \tfrac{1}{2}O_2 \rightarrow 2 \left[\begin{array}{c} CH_2S- \quad NH_3^+ \\ | \qquad\qquad | \\ CH_2-N=CCH_3 \end{array} \right]$$

(II)
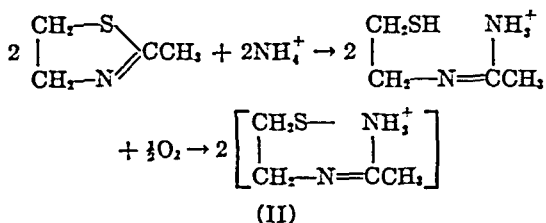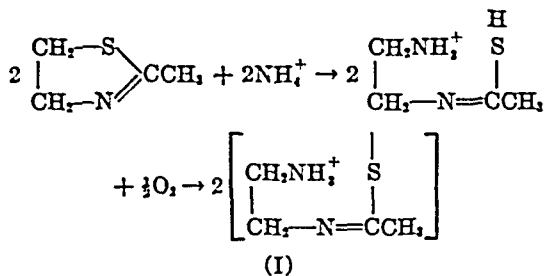
In scheme (II) the strong base amidine is formed, while in scheme (I) we have to do with an ordinary primary amine.   Hence by titration in alcohol it is possible to distinguish between (I) and (II).   On titration in 90 per cent alcohol, with thymolphthalein as indicator, the yield was 64 per cent of theoretical; with phenolphthalein as indicator, 35 per cent of theoretical. The end-point was very unsharp in both titrations, so the values are only approximate.   There is no doubt, however, that the substance is a strong base and that, consequently, scheme (II) must be adopted.

The amidine is very stable in acid solution.   Even after 24 hours boiling with 5 N HCl the typical picrate could be isolated from the hydrolysate in abundant quantities.   In faintly basic solutions it is relatively stable but in 2 N NaOH at 100° half of its nitrogen is fairly rapidly split off as ammonia.

The reaction between thiazoline and ammonium ions is not instantaneous but proceeds at a measurable rate, the velocity constant, K_R, for the bimolecular reaction being around 0.02

in 5.78 N guanidine bromide.   The process does not run to completion but a state of equilibrium is reached depending upon the concentrations of the reactants as well as upon the salt concentration of the reaction mixture.   As distinct from the process of hydrolysis which is mostly inhibited by salts, the amidine formation (x being a substituted amidine) is promoted in a strong salt solution and, as *in the case of protein denaturation* (*Greenstein* (8)), *an increasing effect is observed in the sequence sulfate, chloride, bromide.*   Iodides and thiocyanates could not be investigated
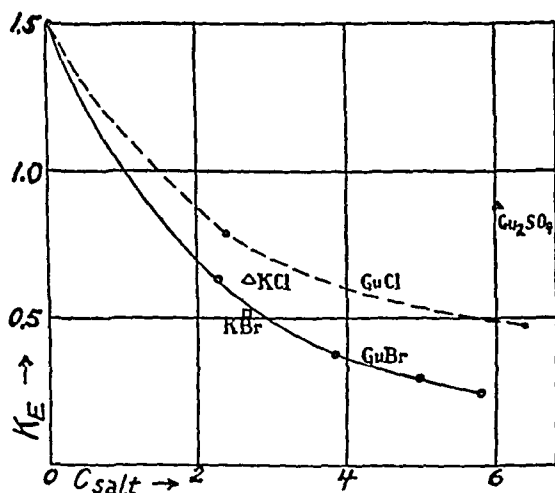
FIG. 3. Equilibrium between thiazoline and ammonium ions in guanidine sulfate, guanidine chloride, guanidine bromide, KCl, and KBr.

because they interfered in the determination of the thiol groups. Fig. 3 shows the variation of the equilibrium constant K_E

$$K_E = \frac{C_{Th} \cdot C_{NH_4^+}}{C_x}$$

as a function of the salt concentration (guanidine sulfate, chloride, and bromide, KCl, and KBr).

K_E is independent of pH within the range of acidity chosen for the experiments; *viz.*, around the neutral point.   From this fact we may only conclude that the reaction either proceeds between uncharged thiazoline molecules and ammonium ions or between thiazoline ions and ammonia molecules.   We have chosen the

first reaction scheme as the simplest one because the concentration of the reactants is higher in this case, but there is a slight possibility that the second reaction actually occurs, since in the reaction with water it is the thiazoline ion which is unstable. This means, however, that K_R has to be multiplied by about 10^4, which makes this constant astonishingly high as compared with that of water. Unfortunately the problem cannot be solved at present.

From the values found for K_E we may calculate the concentration of x at any given concentration of thiazoline and ammonium ions. Thus for $C_{Th} = 0.1$ and $C_{NH_4^+} = 0.1$ we find $C_x = 0.0025$ in 5.78 M guanidine bromide; i.c., only 2.5 per cent of the thiazoline present is transformed into amidine in this case.

Besides ammonium ions, primary amine ions such as the ions of methylamine or glycine will react with thiazoline. In the case of methylamine ions the equilibrium constant is but slightly higher than that found for ammonium ions; viz., 0.33 against 0.24 in 5.78 M guanidine bromide. The corresponding constants for the dimethylamine and trimethylamine ions are, however, much higher; indeed, the reaction was so faint and set in so rapidly that we suspect the presence of a small impurity in the salts applied. Hence it would seem that the group $R—NH_3^+$ is necessary for the reaction to occur, which also agrees with the fact that urea is without effect.

The preferential position of NH_4^+ and $R—NH_3^+$ in the reaction with thiazoline may be understood on the basis of both of the reaction schemes (I) and (II). It is quite clear that the substance (y) cannot be formed unless the ion reacting with thiazoline is able to give off 2 hydrogen atoms. In scheme (I) we may consider the possibility of tautomeric displacement of the double bond
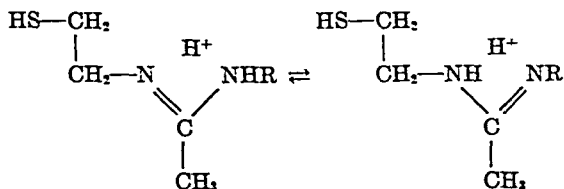
to be a necessary condition for the reaction to occur. Here we understand better why the amine has to be basic.

The behavior of the guanidine ion is still obscure.   There is no doubt that the equilibrium constant is exceedingly high.   On the other hand we have indications that porphyrindin reacts much more rapidly with thiazoline in guanidine bromide solutions than may be explained by the hydrolysis.   The question is being further investigated.

A closer study of the relation of these results to the problem of protein denaturation is of course impossible at the present moment.   More experiments have to be made with more complex thiazoline compounds; viz., compounds of the general structure

$$\begin{array}{c} CH_2\!-\!S \\ | \qquad\qquad \diagdown \\ \qquad\qquad\qquad CCH_2NHCOR_2 \\ | \qquad\qquad \diagup \\ CH\!-\!N \\ | \\ CONHR_1 \end{array}$$
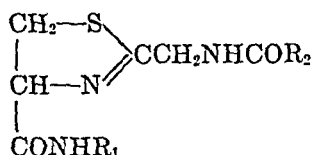
which may be formed by folding up a peptide chain

$$\begin{array}{ccc} CH_2SH & & CH_2\!-\!S\boxed{HHO} \\ | & & | \qquad\quad | \\ R_1NHCOCH\!-\!NHCOCH_2NHCOR_2 & \rightarrow & R_1NHCOCH\!-\!N\!\!=\!\!CCH_2NHCOR_2 \end{array}$$
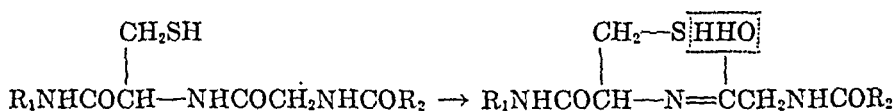
It is not unlikely, however, that our results may sometime or other become of value for the study of protein denaturation.   The peculiar instability of the thiazoline ring, the reversibility of the reaction with ammonium ions, the fall in the effect from bromide via chloride to sulfate solutions which is also found in the case of proteins (8), all point in this direction.   We especially consider the latter analogy important, because it indicates a similar structure in the reaction products formed in the two cases.

The rôle played by the anions is rather obscure but on the basis of the equation for the activity coefficients

$$\frac{f_{NH_4^+}\cdot f_{Th}}{f_x} = \frac{(K_E)\ \text{water}}{(K_E)\ \text{salt solution}}$$

we may conclude that the activity coefficient of the substance (x), the amidine, decreases strongly when we pass from sulfates via chlorides to bromides.   The other possibility, that the product $f_{NH_4^+}\cdot f_{Th}$ increases in the same sequence, is hardly realized, because few cases are known in which bromides have a greater salting-out

effect than chlorides. Actually, a pronounced resemblance is found between the above system and the equilibrium system, quinone + H$_2$ ⇌ hydroquinone, which was investigated by one of the authors (17). Here it was proved that the activity coefficient of quinone decreases with increasing atomic volume of the anion, while the activity coefficient of hydroquinone (and that of hydrogen) is but little influenced by a replacement of one anion by another. It would be worth while investigating whether the resonance in the amidine molecule mentioned in the theoretical part may be of importance for this specific effect of the anions. Possibly some light would be thrown upon the properties of quinone and of proteins as well. We cannot but think that some key to the protein problem is concealed in this peculiar salt effect.

The idea that ring structures are present in protein molecules is nearly as old as the peptide chain theory, and, as mentioned in the beginning, this particular ring structure was proposed in 1924 by Bergmann who used the oxazoline ring as an example. Unfortunately the oxazolines are much more difficult to study, because easy methods for determining hydroxyl groups are not at hand. But preliminary investigations seem to show that oxazolines react in a similar way to thiazoline.

We do not believe, of course, that an opening up of thiazoline-like rings is the sole reason for the complete destruction of the protein molecule in the denaturation. Above all, the quantity of such bonds is too small to make the idea likely. But taking oxazoline rings into consideration, too, may possibly lead somewhere.

To our knowledge the effect of ammonium ions on proteins has not been studied extensively. Since proteins contain free amine groups, the denaturation by KI or KSCN might be explained as an amidine formation. We shall further refer to a note by Edsall, Greenstein, and Mehl (18) in which it was stated that ammonium and methylamine ions cause the —SH groups of myosin to disappear simultaneously with the disappearance of double refraction of flow. It is not easy to bring this phenomenon into direct connection with the results of the present paper but we may point out that a formation of compounds such as the hypothetical compound (y) may furnish an explanation of this phenomenon. The processes represented in the formulas are
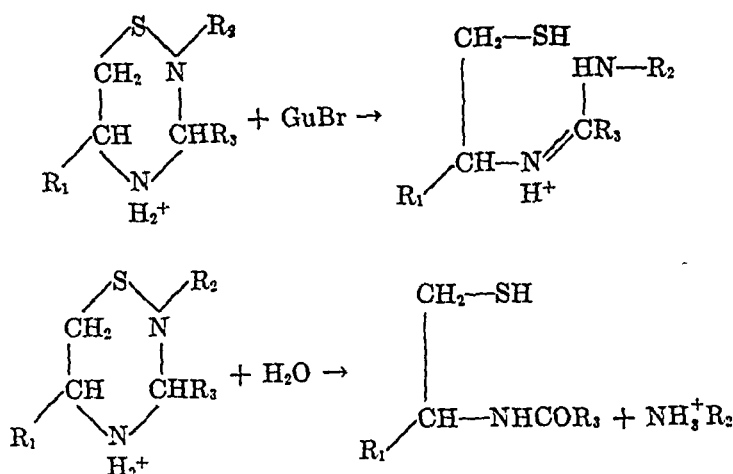
not impossible either as elements to be taken into consideration in the mechanism of denaturation. They are not incompatible with our experiments, since we have been unable to decide whether one or the other or both of the substances (x) and (y) are formed in the reaction with amine ions. Hence it may be worth while to keep compound (y) in mind as a structural unit in the protein molecule.

<div align="center">SUMMARY</div>

We may sum up the results of our investigations as follows: 2-Methylthiazoline is an unstable compound which is easily "denatured" by heat in acid solutions, and by the specific agents ammonium chloride and bromide. As a result of the "denaturation" thiol groups appear. A peptide bond (which may be split by a suitable enzyme) is formed in the case of heat "denaturation."

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LETTERS TO THE EDITORS

BIOTIN AS A GROWTH FACTOR FOR C203S STRAIN OF HEMOLYTIC STREPTOCOCCUS, GROUP A*

Sirs:

Biotin has been reported as an essential growth factor for several microorganisms: *Bacterium radicicola*,[1] *Clostridium butylicum*,[2] other strains of *Clostridia* including *Clostridium sporogenes*,[3] and recently for some strains of *Staphylococcus aureus*.[4]

In a study of the nutrition of the C203S strain of Group A hemolytic streptococcus,[5] we found that a biotin concentrate was necessary for growth. Through the courtesy of Dr. W. H. Peterson we have since had the opportunity to test a sample of crystalline biotin sent him by Dr. F. Kögl.[6]

Experiments were carried out on a hydrolyzed gelatin medium similar to that already described,[5] and on a medium prepared by substituting the following amino acids for the hydrolyzed gelatin: 100 mg. each of *dl*-threonine, *dl*-serine, *dl*-isoleucine, *dl*-leucine, *dl*-valine, *dl*-glycine, *dl*-methionine, *dl*-lysine, *dl*-proline, *d*-alanine, *d*-arginine, *l*-histidine, *l*-tyrosine, *l*-tryptophane, and 200 mg. each of *dl*-cystine and *dl*-phenylalanine.

The inoculum consisted of 1 drop of 6 to 8 hour broth culture of C203S which was centrifuged, washed twice with saline in the

[1] Nilsson, R., Bjälve, G., and Burström, D., *Naturwissenschaften*, **27**, 389 (1939).

[2] Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, **61**, 3594 (1939).

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[4] Porter, J. R., and Pelczar, M. J., *Science*, **91**, 576 (1940).

[5] Pappenheimer, A. M., Jr., and Hottle, G. A., *Proc. Soc. Exp. Biol. and Med.*, **44**, 645 (1940).

[6] Kögl, F., *Z. physiol. Chem.*, **242**, 43 (1936).

centrifuge, and then made up to slight turbidity with saline. Tubes were incubated under 12 mm. of CO_2 tension in air for 16 hours, then at atmospheric CO_2 tension for an additional 24 hours. The amount of growth was determined with the photoelectric turbidometer and the readings were correlated with standard suspensions of known bacterial nitrogen content.

Effect of Biotin on Growth of C203S Strain

Biotin added to 10 cc.	Bacterial nitrogen from 10 cc. × 50	
	Hydrolyzed gelatin medium	Amino acid medium
γ	*mg.*	*mg.*
0.002	13.5	10.3
0.0008	13.6	11.0
0.0004	12.2	9.6
0.0002	10.0	8.6
0.0001	6.3	7.7
0.00005	3.1	6.4
0.000025	2.0	3.0
0.0	1.4	1.4

The figures are the average of duplicate determinations.

The results confirm our previous suggestion that biotin is an essential growth factor for the C203S strain of Group A hemolytic streptococcus. The accompanying table shows that maximum growth is obtained on the addition of 0.08 γ of crystalline biotin per liter of deficient medium.

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CHYMOPAPAIN: A NEW CRYSTALLINE PROTEINASE FROM PAPAYA LATEX*

Sirs:

In the preparation of crystalline papain[1] it was shown that the yield of crystalline enzyme represented only a minor part of the total proteolytic activity of fresh papaya latex. It was furthermore demonstrated that the residue of uncrystallized protein, the so called "high salt fraction," contained another enzyme with a higher ratio of protein-digesting activity to milk-clotting power than that of crystalline papain.

Observations made on the same "high salt fractions" recently showed that at 10° the proteolytic action was undiminished for several weeks at pH 2. This observation provided a means whereby the enzyme could be prepared in the pure state.

The addition of hydrochloric acid to a suspension of coagulated undried papaya latex in an amount sufficient to produce a pH of 1.8 to 2.0 caused an extensive precipitation of protein. The protein remaining in solution was, however, proteolytically active. An inert fraction of this protein was found to be precipitable by half saturation with sodium chloride after the addition of sodium hydroxide had increased the pH to 3.5 to 4.0. The remaining protein was not precipitated by complete saturation with NaCl at pH 4.5, but was completely precipitated from saturated NaCl solutions at pH 2.0. It was thus possible to precipitate the protein in two ways; by adding NaCl to a half saturated NaCl solution at pH 2.0 or thereabout or by adding HCl to a solution saturated with NaCl at pH 4.5 to 6.0. Both methods produced obviously crystalline material. The best crystals were obtained by a compromise between them. Protein recrystallized four times was proteolytic and also active as a milk-clotting agent,

[1] Balls, A. K., and Lineweaver, H., *J. Biol. Chem.*, **130**, 669 (1939).

but it was observed to differ in several ways from the crystalline papain originally prepared in this Laboratory.

The new crystalline enzyme occurred in broad sabre-like needles, and sometimes in obvious plates. Its proteolytic activity was furthermore stable at pH 2.0 when the temperature was 10°. The crystals without preliminary denaturation or the addition of urea or treatment with cyanide gave a strong —SH test with nitroprusside.

The newly crystallized enzyme is many times more soluble than the previously described crystalline papain at salt concentrations and pH at which the latter is soluble.

The activity of the newly isolated enzyme when computed per unit of protein nitrogen was found to be equal to that of crystalline papain with respect to milk clotting, and half that of crystalline papain with respect to hemoglobin digestion. The ratio of milk-clotting activity to hemoglobin digestion was found to be exactly twice that of crystalline papain.

Because of the property last mentioned and by reason of the precedent set by Kunitz and Northrop[2] in naming chymotrypsin, we wish this new crystalline protein to be known as *chymopapain.*

The quantity of chymopapain present in crude latex is considerably greater than is that of papain. Its preparation in comparatively pure state, without crystallization, is easy; its stability in acid may prove to be an important pharmaceutical and technical asset. The Secretary of Agriculture has kindly consented to apply for a patent on this material for the use of the public.

Enzyme Research Laboratory   E. F. JANSEN
  Bureau of Agricultural Chemistry and   A. K. BALLS
    Engineering
  United States Department of Agriculture
  Washington

² Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **18,** 433 (1935).

THE SOLUBILITY AND TITRATION OF HEMIN AND FERRIHEMIC ACID*

By DEMPSIE B. MORRISON AND EDWARD F. WILLIAMS, JR.

(From the Department of Chemistry, University of Tennessee College of Medicine, Memphis)

In 1929 Fischer and Zeile (2) accomplished the synthesis and established the formula of hemin. Ferrihemic acid is related to hemin as shown in the accompanying formulas. In each com-
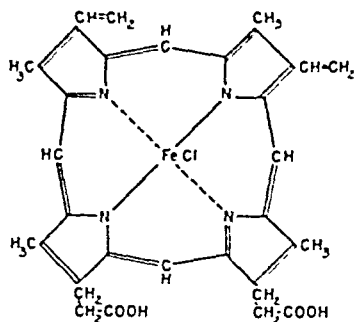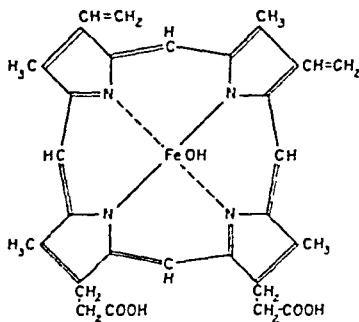
Hemin   Ferrihemic acid

pound we have essentially a symmetrical dibasic acid and a basic iron group. The chloride group which is attached to iron in hemin has been replaced by hydroxyl in ferrihemic acid, the molecular weights being, respectively, 652 and 633. Pauling and Coryell (3) have shown that "the iron atom is attached to the four adjacent nitrogen atoms of the porphyrin not by covalent but by ionic bonds; that is, iron is present essentially as ferric ion in ferriheme."

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* As a more appropriate designation of the compound which has been variously called *acid hematin, ferrihemic hydroxide,* etc., and to facilitate reference to its salts, we propose the name *ferrihemic acid.* Accordingly, the salts are called ferrihemates.

A preliminary report of this study has appeared (1).

In view of the relatively symmetrical shape and large size of the molecules it may be expected that the two carboxyl groups will have approximately equal ionization constants and will form dibasic rather than monobasic salts. Hamsik (4), in 1924, prepared crystalline dipotassium ferrihemate from hemin by the action of dilute potassium hydroxide in ethanol.

Both hemin and ferrihemic acid are exceedingly insoluble in water, a fact which probably accounts for the observation of Hamsik (5) that hemin is not appreciably hydrolyzed to ferrihemic acid by boiling water. However, when hemin dissolves in alkaline solution, the chloride group is replaced by hydroxyl, ferrihemate being formed.

Hogness et al. (6) and Haurowitz (7) have shown that alkali ferrihemates polymerize, polymerization being favored as the pH and the concentration are increased.

Fischer and Orth (8) have summarized to 1937 the extensive studies which have been made of oxidation-reduction potentials and derivatives of hemin and ferrihemic acid, including their esters, hemochromogens, and inorganic salts. Data of solubilities and relative strengths of acid and basic groups appear to be entirely lacking. Barron (9) obtained erratic values for the oxidation-reduction potentials of ferrihemates at pH below 7.

The solubilities of ferrihemates are important for both chemical and physiological application. As a continuation of our earlier studies of hemoglobin (10–12) and as a necessary preliminary to further chemical, physiological, and metabolic[1] studies of the ferrihemates, we have measured their solubilities, obtained an approximation of relative strengths of their acid and basic groups, and determined the over-all charge on the ferrihemate ion at widely different pH levels.

---

[1] While we expect to report subsequently and in detail observations of the metabolism of the ferrihemates, we may note here, as relevant to the present paper, that dogs will tolerate injections of reasonably large amounts of ferrihemates. Ferrihemate when injected in solution appears to remain in solution in the blood for some time. As ferrihemate disappears from the blood, it may be found, apparently as a derivative, in insoluble form, mainly in the reticulo-endothelial tissue. Brown (13) has reported that hemin crystals placed in the peritoneal cavity did not dissolve but became encapsulated and remained in this condition for some weeks.

Preparation of Hemin—Hemin crystals were prepared from washed erythrocytes of the pig, ox, or dog by the method of Nencki and Zaleski (14) as modified by Drabkin and Austin (15), and were recrystallized in some cases by the procedure of Conant (16) or Schalfejew (17). The crystals were dried to constant weight in air at 40°.

Preparation of Ferrihemic Acid—Ferrihemic acid when first precipitated forms a voluminous gelatinous mass resembling ferric hydroxide. Since in this form it is difficult to wash free of chloride, it has been prepared in small lots to facilitate purification. 2 gm. of hemin crystals are dissolved in 90 ml. of 0.12 N NaOH. This solution is diluted to 3500 ml. with distilled water. Ferrihemic acid is precipitated by the slow addition, with constant stirring, of 100 ml. of 0.118 N HCl. It is necessary to make the solution more acid than pH 4 to liberate all base, although precipitation is complete at about pH 6. After standing overnight the clear supernatant liquid is siphoned off and the precipitated acid, in volume about 650 ml., is transferred to centrifuge cups and washed with water by centrifugation until the washings are chloride-free or until the ferrihemic acid begins to be colloidally dispersed. *This tendency appears as electrolyte is removed.* After further washing, once each with 65 per cent ethanol and diethyl ether, the gelatinous precipitate still has a volume of about 150 ml. The precipitate is then dried in air at 40°. The volume of the dried material is now only about 2 ml. A small amount of chloride is still present.

Ferrihemic acid when first precipitated is a strongly hydrophilic colloid and retains water tenaciously, as shown by the volume changes in the above procedures. One sample, washed with water only, contained 0.75 per cent chloride, while another sample which was partially dehydrated by further washings with ethanol and ether contained only 0.29 per cent chloride. The theoretical chlorine content of hemin is 5.44 per cent. The small residues of chloride may represent adsorption on the voluminous precipitate of ferrihemic acid or the regeneration of a small amount of hemin. Part of the chloride, at least, is probably traces of adsorbed HCl, since the pH values of all ferrihemic acid samples in water were somewhat lower than for the hemin samples; 0.6 mM of alkali

per liter failed to dissolve detectable amounts of the ferrihemic acid, whereas in this concentration of alkali some solution of hemin occurred in five of six samples.

In the preparation of one sample of ferrihemic acid just enough HCl was added to precipitate completely the insoluble acid, which was then washed and dried in the usual manner. Titration and solubility curves constructed from this sample showed that, despite the repeated washings, approximately 23 per cent of the base was retained.

Preparation of Dipotassium Ferrihemate—Dipotassium ferrihemate was prepared by dissolving hemin crystals in a small volume of 0.1 N KOH and then adding solid KOH, with motor stirring, until the salt separated. The solution was kept at room temperature by being cooled with running water. The salt was collected by centrifuging; the supernatant liquid was discarded and the salt was washed free of excess base with 95 per cent ethanol which had been distilled from KOH to remove any acids. The salt was dried in air at 40°. As thus obtained, the salt is readily and completely soluble in water. While we have not determined maximum solubility, a 3.3 per cent solution was readily prepared.

Solubility and Titration of Hemin and Ferrihemic Acid—Solubility and partial titration curves have been constructed from data obtained in the following manner. 0.350 gm. samples of crystalline hemin or ferrihemic acid powder were weighed into 30 ml. screw-cap bottles. To the first bottle in each series were added 20 ml. of distilled water. To other bottles were added sufficient distilled water and 0.1200 N NaOH or 0.1065 N KOH to make a final volume of exactly 20 ml. and a final concentration of added alkali from 0.6 to 90 mM per liter (in a few samples the concentration of alkali was up to 120 mM per liter). In some cases the alkali was added first and then diluted to 20 ml. with distilled water; this order tended to give a higher pH and greater solubility, particularly with the lower concentrations of alkali. When solution was complete, the hemin concentration was 26.8 mM, and the ferrihemic acid concentration was 27.6 mM, per liter.

The bottles were capped and rotated, end over end, for from 3 to 8 days in a constant temperature air bath at 30°, and then were centrifuged at high speed to separate any undissolved mate-

rial. In aliquots of the supernatant liquid dissolved ferrihemate and the pH were determined.

All pH measurements were made with the glass electrode at room temperature which was maintained constant ±0.5° for any one series. The average temperature, and the temperature at which most measurements were made, was approximately 25°. The concentration of dissolved ferrihemate was determined, after suitable dilution of the sample with 0.036 N alkali, with a spectrophotometer of the König-Martens type at 515 mμ wavelength. Standardization of the acid and alkalies has been described (10).

Seven series of determinations were made with hemin, of which four were with hemin crystals and NaOH, two with recrystallized hemin and NaOH, and one with recrystallized hemin and KOH. Five series were completed with ferrihemic acid and NaOH; one of these was discarded, since in preparing the ferrihemic acid from its salt all base was not removed (see description of preparation above). One sample of hemin crystals was equilibrated with a series of solutions of HCl ranging in concentration up to 118 mM per liter. The crystals did not dissolve and had no buffering effect. Although the statement has been made that hemin is soluble in dilute acids, we have found that hemin does not dissolve in HCl at any concentration which does not split iron from the molecule.

In all hemin experiments crystals of hemin with their characteristic shape and color remained undissolved at all concentrations of alkali up to about 90 per cent of that required for complete conversion to salt.

Fig. 1 records all of the solubility and titration data obtained in the seven series of hemin determinations. The individual points which represent titration values (upper curve) are plotted as mM of base per liter against the pH scale. The titration curve as drawn, however, is an average of the individual curves of the several series. This is preferred to a curve constructed from an average of individual points, since the latter do not represent a consistent order of variation of base concentrations in the different series.

In the lower part of Fig. 1 the points represent the solubilities of hemin plotted as mM per liter against mM of added base. The

curve of solubility as shown is drawn to the theoretical slope of 1 mole of hemin dissolving for each 3 moles of added alkali. This curve is terminated at a point which represents 26.8 mM of hemin and 80.4 mM of base, where, theoretically, solution should be complete. A line drawn from this point parallel to the pH axis would intersect the upper, titration, curve at pH 9.7, approxi-
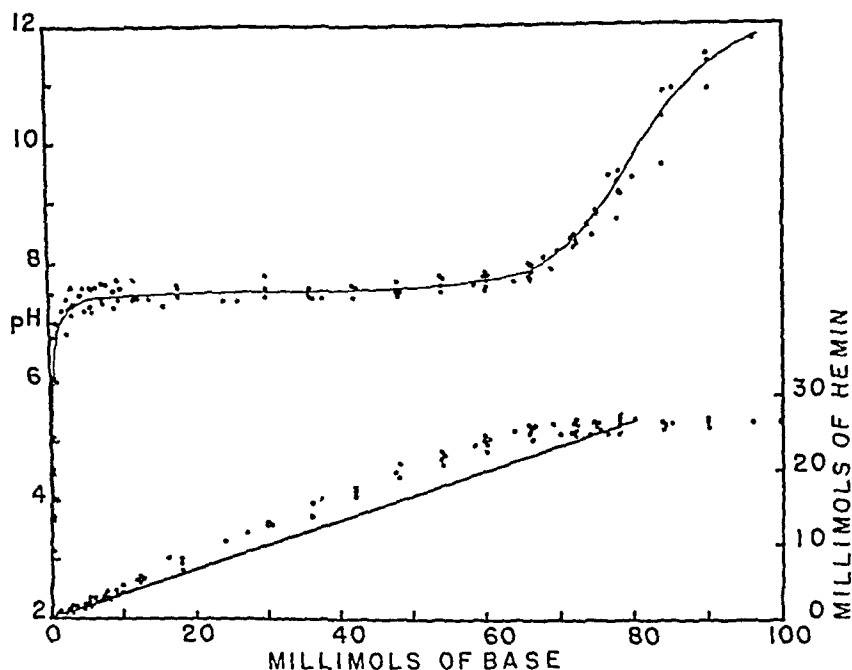
FIG. 1. Titration and solubility of hemin. Titration data (upper curve), plotted as mM of base against pH, of seven hemin series, six with sodium hydroxide and one with potassium hydroxide. The solubility data (lower curve) are plotted as mM of base against mM of hemin. The slope and length of the line represent the theoretical for the conversion of 26.8 mM of hemin to disodium ferrihemate or 3 equivalents of base per mole of hemin. An extension of this point upward parallel to the pH axis intersects the titration curve at approximately pH 9.7.

mately. This pH, therefore, should characterize a solution of alkali ferrihemate in which the 2 carboxyl hydrogen atoms have been replaced by univalent base and the chloride has been replaced by hydroxyl. Our results confirm that 3 equivalents of base are required to convert 1 mole of hemin to 1 mole of ferrihemate. The fact that hemin appears to dissolve in lower concentrations of

alkali to an extent somewhat greater than theory predicts is to be explained, we believe, by the tendency to form a colloidal dispersion. This explanation is supported by the results of titrations of disodium and dipotassium ferrihemates with HCl (below), which also discredit the possibility that some part of the hemin might be converted to the dibasic salt without substitution of the chloride group. Since crystals of hemin persist until approximately 90 per cent of full alkali equivalence has been reached, it is improbable that significant formation of monobasic salt has occurred. Absence of any break in solubility or titration curves is further evidence that hemin is converted to dialkali ferrihemate without formation of monosalt.

Erratic pH values, so conspicuous at the lower end of the titration curve, are, at least in part, due to the fact that the system is poorly buffered, ferrihemate ion being soluble only to the extent of 0 to 5 mM per liter through this pH range.

Fig. 2 summarizes solubility and titration data of four series of ferrihemic acid determinations. The solubility (lower) curve again represents theoretical alkali equivalents, 2 equivalents of base being required to convert ferrihemic acid to dialkali hemate, since in this case there is no chloride to replace. The extent of deviation of this curve from experimentally determined values is of the same order as in the hemin experiments and, presumably, for the same reasons. The curve ends at a point which represents 27.6 mM of ferrihemic acid and 55.2 mM of base per liter. The corresponding point on the titration curve coincides with a pH of 9.0, approximately.

At the lower end of the titration (upper) curve in Fig. 2, pH values are erratic as in the hemin series, both because of poor buffering on account of the negligible solubility of ferrihemic acid through this range and because the ferrihemic acid samples contained traces (not more than 1 mM per liter or less than 2 per cent) of acid (probably HCl).

Titration of Disodium Ferrihemate—Stock solutions of disodium ferrihemate were prepared by dissolving hemin crystals in 0.036 N or 0.120 N NaOH. The ferrihemate concentrations of the stock solutions ranged from 6.0 to 40.0 mM per liter. Five series of titrations were made at constant volume, the procedure being as follows: Of a stock solution 10 ml. aliquots were pipetted into a

series of screw-cap bottles. To the first bottle of the series were added 10 or 20 ml. of distilled water, and to the other bottles of the series the same volume of standard HCl solutions of progressively increasing concentrations. Thus, in any one series, the final volumes in all bottles were the same, the amounts of added hemin and alkali fixed, but the amounts of added HCl varied in progressive order from bottle to bottle.
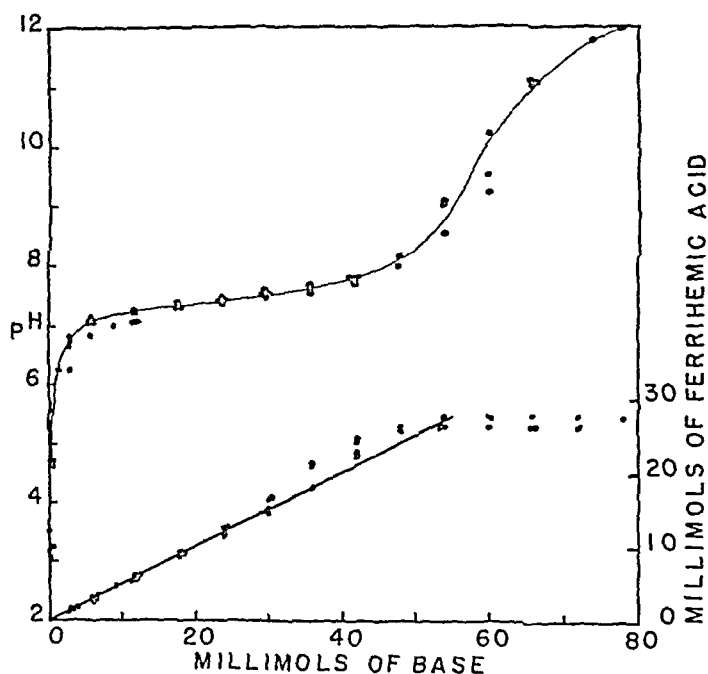
Fig. 2. Titration and solubility of ferrihemic acid. Titration data (upper curve), plotted as mM of base against pH. The solubility data (lower curve) are plotted as mM of base against mM of ferrihemic acid. The slope and length of the line represent the theoretical for the conversion of 27.6 mM of ferrihemic acid to disodium ferrihemate or 2 equivalents of base per mole of acid.

In a sixth series, 10 ml. aliquots of a solution containing per liter 21.5 mM of disodium ferrihemate in 0.120 N NaOH were titrated with 0.118 N HCl, the added volumes of the latter ranging from 0 to 12 ml.

The bottles were rotated in an air bath at 30° for from 2 to 7 days and were then centrifuged. In the supernatant fluid the pH was measured and the dissolved or colloidally dispersed pigment was determined spectrophotometrically.

One series of determinations was made of solubility of hemin crystals in 0.067 M phosphate buffers of varying pH.

In these titrations the amounts of NaCl formed by interaction of hemin with NaOH ranged from 3.0 to 20.0 mM per liter. Additional NaCl formed from added HCl ranged from 0 to 40.0 mM per liter in the five constant volume series.

Precipitation was complete in the seven series, including the phosphate series, when the pH had been lowered to 5.95 ± 0.13. In the most dilute ferrihemate solutions containing a large excess of alkali, and thus providing on neutralization a relatively large ratio of NaCl to ferrihemate, approximately 2 equivalents of HCl were required to effect complete precipitation. Since, in theory, 2 equivalents of HCl convert dialkali ferrihemate to ferrihemic acid rather than hemin (owing to hydrolysis of the chloride group of hemin through the pH range studied), the precipitate here is, presumably, almost entirely ferrihemic acid. In more concentrated ferrihemate solutions no precipitation occurred until approximately 1 equivalent of HCl had been added. At this point, further addition of a small amount of HCl (less than a 2nd equivalent; cf. Fig. 3) tended to cause abrupt precipitation. It appears that when not more than 50 per cent, approximately, of the ferrihemate has been converted to ferrihemic acid the latter is held in colloidal dispersion by the residual ferrihemate; with further conversion, the free acid precipitates abruptly, carrying with it by adsorption the remaining ferrihemate. In the more dilute ferrihemate solutions with relatively larger proportions of NaCl, colloidal dispersion of ferrihemic acid is avoided.

As noted previously, freshly precipitated ferrihemic acid is gelatinous and powerfully hydrophilic. It is in this form that ferrihemic acid is precipitated in titrations of alkali ferrihemate. This, we believe, may account for the discrepancies between these titrations and the reverse titrations of hemin or dried ferrihemic acid with NaOH.

The pH falls smoothly in these series with increased addition of HCl; there is no sudden break in the titration curve when precipitation occurs, but only when 2 equivalents of HCl have been added. At this point the basic iron group begins to titrate.

In Fig. 3, Curves 1 and 2 represent, respectively, the titration of 14.8 and 20.0 mM solutions of disodium ferrihemate. Corresponding solubility curves (Curves 1-a and 2-a) are also shown in

Fig. 3. The 14.8 mM solution had been equilibrated for 7 days; the 20.0 mM solution for 2 days. The curves emphasize the difficulty of attaining solubility and pH equilibria in titration of alkali ferrihemate, and the relative weakness of the basic iron group. It is apparent that even 7 days do not suffice for complete equilibration.

Titration of Dipotassium Ferrihemate—Aqueous solutions of dipotassium ferrihemate were prepared from two samples of the pure salt. Of one sample, 1.652 gm. were dissolved in 50 ml.
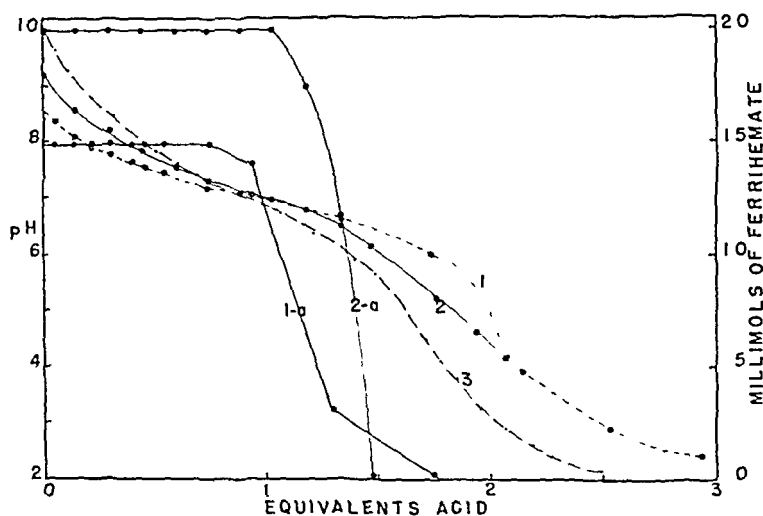
FIG. 3. Curves 1 and 2 represent the titration of a 14.8 and a 20.0 mM solution of disodium ferrihemate with hydrochloric acid. Curves 1-a and 2-a represent the corresponding solubility curves; Curve 3, the titration of a 31.2 mM solution of dipotassium ferrihemate. Titration curves are to be read in equivalents of acid against pH and solubility curves in equivalents of acid against mM of ferrihemate.

of distilled water and titrated with 0.200 N acid from a semimicroburette, with stirring by hand. pH values were erratic at occasional points, indicating that hand stirring was not vigorous or prolonged enough between some additions of the HCl. Of the second sample, 2.234 gm. were dissolved in 100 ml. of distilled water (concentration, 31.2 mM per liter) and titrated with 0.326 N HCl. Here, the solution was stirred vigorously by a motor stirrer, the tip of the burette was immersed in the solution, and the acid was added very slowly. It was observed that at pH 6, approximately, precipitation was complete and the bulky, ge-

latinous precipitate filled the vessel. At the concentrations of ferrihemate used the precipitate settles very slowly.

Curve 3 in Fig. 3 represents the second of the above titrations. It is to be noted that all curves of Fig. 3 have the common characteristic of passing through a pH of 7, approximately, when 1 equivalent of acid has been added. Addition of the 2 equivalents of acid which are theoretical for conversion of dialkali ferrihemate brings the curves to pH 4, approximately. Titration of the basic iron group by a 3rd equivalent of acid is in the lower pH range; it is indicated that the iron group is 50 per cent neutralized at about pH 3, corresponding to a pK value of about 11 for this group.

Cataphoretic Measurements—Solutions of disodium ferrihemate in dilute NaOH, or in phosphate buffer, were placed in a Y-tube, the ends of two of the legs being covered with membranes cut from viscose casing. These ends were then immersed in liter beakers containing NaOH solution or phosphate buffer of the same concentration as that used to dissolve the hemin. A direct current of 450 volts and approximately 50 milliamperes was employed. The ferrihemate ion migrated to the anode, collecting in a concentrated layer about 12 mm. thick. Reversal of the current reversed the direction of migration. In ethanol solution, containing a small amount of hydrochloric acid, hemin moved much more slowly but always to the anode. Thus, the over-all charge of the ferrihemate ion, or of hemin in acid alcohol, is negative.

DISCUSSION

It is clear from our data that ferrihemic acid requires for its conversion to a soluble dibasic salt 2 equivalents of base, whereas hemin requires 3 base equivalents. Absence of a break in the titration and solubility curves of ferrihemic acid and hemin indicates that the two carboxyl groups are approximately equally ionized, so that monobasic salt is not formed in detectable amount. This behavior is consistent with the formulas. In this behavior ferrihemic acid and hemin are comparable to succinic acid and its higher homologues.

The very small solubility of ferrihemic acid and hemin is evidenced in the relatively flat shape of the titration curves.

If the titration curves of ferrihemic acid and hemin are plotted on a per cent neutralization scale, with 100 per cent neutralization

representing 2 equivalents of base for ferrihemic acid and 3 equivalents of base for hemin, the curves will superimpose except through the lower pH range where the ferrihemic acid curve falls somewhat below the hemin curve. It would appear that the chloride group of hemin titrates or hydrolyzes simultaneously with the formation of the dibasic salt, and that salt formation (solution) is prerequisite for the hydrolysis. This conclusion is supported by our method of preparing ferrihemic acid, and is in agreement with the observation of Hamsik (5). The reactions are as follows, where $(Por) = C_{32}H_{30}N_4$:

$$(HOOC)_2(Por)FeOH + 2NaOH \longrightarrow (NaOOC)_2(Por)FeOH + 2H_2O$$

Ferrihemic acid   Disodium ferrihemate

$$(HOOC)_2(Por)FeCl + 3NaOH \rightarrow (NaOOC)_2(Por)FeOH + 2H_2O + NaCl$$

     Hemin

The titration curves represent an equilibrium between difficultly soluble acids and their soluble salts. Over the pH range required for titration and solution of ferrihemic acid, and in the absence of chloride, ionization of the —FeOH group is negligible. Thus, ionization of the carboxyl groups, only, is to be considered:

$$(HOOC)_2(Por)FeOH \leftrightharpoons H^+ + (-OOC)(HOOC)(Por)FeOH^- \leftrightharpoons H^+ +$$
$$(-OOC)_2(Por)FeOH^-$$

Hence,

$$\frac{[H^+]^2 \times [(-OOC)_2(Por)FeOH^-]}{[(HOOC)_2(Por)FeOH]} = K_{ionization}$$

And since, because of the great insolubility of the acid, we may assume complete ionization in the saturated solution,

$$[H^+]^2 \times [(-OOC)_2(Por)FeOH^-] = S \text{ (ion product constant)}$$

The simplified equation, where C is the salt concentration in moles per liter, is

$$pH = \tfrac{1}{2}\log C - \tfrac{1}{2}\log S$$

which rearranges to

$$\text{Log } S = \log C - 2pH$$

Employing the last equation, we have calculated from our data the values of S and the solubility of hemin and ferrihemic acid

over the more effectively buffered portions of the curves which represent 20 to 90 per cent of neutralization. For the four ferrihemic acid series, $\log S = -16.76 \pm 0.04$ (probable error of the mean), $S = 0.174 \times 10^{-16}$, and solubility $= 1.63 \times 10^{-6}$ mole or 0.0010 gm. per liter. For the seven hemin series, $\log S = -17.00 \pm 0.03$ (probable error of the mean), $S = 1 \times 10^{-17}$, and solubility $= 1.36 \times 10^{-6}$ mole or 0.0009 gm. per liter.

SUMMARY

The ion product constants and solubilities of hemin and ferrihemic acid have been determined.

3 equivalents of alkali are required to dissolve hemin, with formation of soluble dialkali ferrihemate and alkali chloride. Only 2 equivalents of alkali are required to dissolve ferrihemic acid. Likewise, in an aqueous solution of dialkali ferrihemate, 3 equivalents of hydrochloric acid precipitate ferrihemic acid and not hemin.

Ferrihemic acid and hemin form with alkali only dibasic and not monobasic salts.

The over-all charge of the ferrihemate ion is negative.

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EXPERIMENTS ON THE CARBOXYLASE OF PEA ROOTS

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It is known that vitamin B_1 is a growth factor for numerous bacteria and fungi including the yeasts (see the summary in Koser and Saunders (1938)). It has also been demonstrated that vitamin B_1 is essential for the growth of the isolated roots of higher plants (Bonner, 1937; Robbins and Bartley, 1937). Because of this general vitamin B_1 requirement of living organisms, it would seem *a priori* probable that the vitamin plays a rôle in some basic cellular process. That this is indeed the case was shown conclusively by the work of Peters and coworkers (see Peters and O'Brien (1938)) and of Lohmann and Schuster (1937). The latter workers found that the prosthetic group of yeast carboxylase is vitamin B_1 pyrophosphate. In the case of yeast, vitamin B_1 is, then, a constituent of a respiratory enzyme and vitamin B_1 pyrophosphate is hence commonly referred to as "cocarboxylase," a terminology used throughout this paper. Although considerable information is available concerning the rôle of vitamin B_1 as a growth factor for roots, there is little known about the carboxylase of such roots. The present work was undertaken with the hope of elucidating possible relationships between vitamin B_1 and the carboxylase of pea roots.

The reaction system under consideration is defined by four experiments which show (1) that the respiration of whole pea roots is affected by the supply of vitamin B_1 in the medium in which the roots are grown (Table I), (2) that pea roots contain an enzyme (carboxylase) capable of decarboxylating pyruvic acid, (3) that by proper treatment of the enzyme it can be made to lose the greater part of its activity and that the activity is restored by

addition of vitamin B_1 pyrophosphate (Tables II and III), (4) that pea roots contain vitamin B_1 pyrophosphate, as determined by the cocarboxylase activity of filtrates of boiled pea root carboxylase with washed yeast. A once precipitated pea root preparation was found to contain approximately $2.1\ \gamma$ of extractable cocarboxylase per gm. of dry weight. These findings indicate that pea roots contain a carboxylase similar in some respects to that found in yeast.

Technical—Preliminary investigation showed that it is essential to extract pea roots at low temperatures if denaturation of the carboxylase is to be avoided. The roots used were from etiolated plants grown for 7 days in coarse sand at 25°. At the end of this time the plants were removed from the sand and cleaned with running water. The roots were then cut off with a pair of scissors

TABLE I

Effect of Vitamin B_1 on Oxygen Consumption of Isolated Pea Roots Grown for 4 Weeks on Medium Containing All Growth Substances Except Vitamin B_1 As Noted

Roots supplied with	O_2 consumption per 100 mg. dry weight in 1 hr.
	c.mm.
No vitamin B_1...	170
Vitamin B_1 (0.1 mg. per liter)...........................	450

and dried superficially. They were next transferred to a large porcelain mortar which was placed in an alcohol-dry ice mixture. After the mass had frozen solidly, it was ground, transferred to centrifuge tubes, and centrifuged for 10 to 20 minutes. The temperature of the material rose to 3–4° during this process. The supernatant liquid was next transferred to a cold flask and the residue extracted with 0.1 M phosphate buffer of pH 6.2. This extract was combined with the preceding supernatant. To each 100 cc. of the combined extract were added 35 gm. of ammonium sulfate. This addition precipitated proteins, among them the carboxylase, which could then be centrifuged off. The supernatant liquid possessed only slight carboxylase activity and was discarded. The precipitated proteins were redissolved in water and again precipitated by ammonium sulfate. Significant losses

of carboxylase activity occurred if the operations were carried out above 5°.

The determinations of carboxylase activity were carried out with Warburg manometers. 2 cc. of the enzyme preparation suspended in phosphate buffer at pH 6.2 and containing 60 to 90 mg. of protein were placed in each Warburg vessel, together with 0.5 cc. of coenzyme solution, containing approximately 100 γ of vitamin B_1 pyrophosphate (Merck). 1 cc. of buffered 0.05 M solution of sodium pyruvate, containing 0.1 per cent $MgCl_2$, was placed in the side arm of the vessel and tipped in after attainment of temperature equilibrium. In some experiments, the coenzyme solution was placed in the side arm, and the pyruvate added with the enzyme. Suitable controls for the CO_2 evolution and for the small O_2 consumption of the preparation were run in every experiment. The CO_2 evolution in the absence of pyruvate was in all cases zero or negligible. In a typical experiment, the CO_2 evolved in 1 hour in the absence of pyruvate was 0.0 c.mm., and in the presence of pyruvate 106 c.mm. Quantitative determinations of vitamin B_1 pyrophosphate were made according to the method of Lohmann and Schuster (1937) with yeast washed with alkaline buffer to remove the coenzyme. It may be noted here that neither the pea root preparation nor the yeast used in these experiments showed increased activity when 100 γ of vitamin B_1 were added with the coenzyme (compare Ochoa and Peters (1938)). Total vitamin B_1 (phosphorylated and unphosphorylated) was determined in a few experiments by the use of the *Phycomyces* assay (Schopfer and Jung, 1937; Bonner and Erickson, 1938). It was found that under the conditions of the assay vitamin B_1 pyrophosphate possessed an activity per mole equal to that of the vitamin itself.

Removal of Prosthetic Group—Several different procedures were tried in attempts to free the pea root carboxylase preparation from a possible prosthetic group. If a once precipitated preparation was boiled and the denatured proteins filtered off, vitamin B_1 pyrophosphate (as determined by its activity in the yeast test) was found in the filtrate. This indicates that the preparation may contain a vitamin B_1 pyrophosphate-protein combination. Washing of the enzyme preparation with alkaline buffer (by suspending the preparation in 0.1 M Na_2HPO_4 and reprecipitating

with ammonium sulfate) had no effect on the pea root enzyme, even though a similar procedure removed coenzyme from yeast carboxylase (Lohmann and Schuster, 1937). Repeated precipitation with ammonium sulfate (eight or more times) or dialysis through a collodion membrane resulted in denaturation of a large part of the enzyme, while the activity of the undenatured portion was not enhanced by addition of vitamin B_1 pyrophosphate. It was not found possible to destroy the activity of the enzyme preparation by treatment with sulfite, although such treatment is known to destroy vitamin B_1 itself (Williams *et al.*, 1935).

The simplest procedure found for reversible inactivation of the enzyme was the action of the enzyme on pyruvate itself. The enzyme preparation, suspended in phosphate buffer of pH 6.2, was added to a 0.05 M pyruvate solution (containing 0.1 per cent $MgCl_2$) in the ratio of 2 volumes of enzyme to 1 volume of pyruvate solution. This mixture was allowed to stand at room temperature for 2 hours and then placed at 0° overnight. Preparations made according to the above procedure, which will be referred to hereafter as "partially inactivated," lost as much as 70 per cent of their original carboxylase activity. Upon addition of crystalline vitamin B_1 pyrophosphate the original activity was completely restored, indicating that no significant denaturation occurred. The presence of pyruvate was essential for the inactivation.

Activating Effect of Pyrophosphate—It was found that vitamin B_1 pyrophosphate can be replaced by pyrophosphate alone in the reactivation of the pea root enzyme. The action of pyrophosphate is not augmented by addition of the vitamin. This is shown in Table II. Similar experiments with washed yeast showed that pyrophosphate is without cocarboxylase effect on our yeast carboxylase.

That pyrophosphate is actually removed in the pyruvate inactivation of the enzyme is indicated by one experiment in which the supernatant fluid (concentrated to a small volume) from the pyruvate treatment for removal of the coenzyme was found to contain no cocarboxylase (was incapable of activating washed yeast), but this concentrated supernatant was still capable of reactivating treated pea root enzyme to some extent, a property possessed by pure pyrophosphate (Table II).

Nature of Pyrophosphate Effect—On the assumption that pea root carboxylase contains vitamin B_1 pyrophosphate as its functional group, it would seem likely from the above that treatment of the enzyme removed only the pyrophosphate, leaving the vitamin B_1 residue bound to the protein carrier in such a manner as to permit a resynthesis upon addition of pyrophosphate. The following experiment bears upon this hypothesis: 160 cc. of raw juice were precipitated once with ammonium sulfate and taken up in 50 cc. of phosphate buffer. This was divided into two portions. One portion, referred to as Fraction I, was immediately assayed for (*a*) carboxylase activity, (*b*) total vitamin B_1 (phosphorylated and unphosphorylated) by the *Phycomyces* method,

TABLE II

Reactivation of Partially Inactivated Pea Root Enzyme

All the flasks contained pyruvate in addition to the indicated reagents.

	CO_2 in 1 hr., c.mm.		
	Experiment 5	Experiment 6	Experiment 16
Nothing added	18.9	29.7	52 4
85 γ vitamin B_1 pyrophosphate	52 4	60 3	
200 γ Na pyrophosphate·10H_2O	40 4		
2000 γ "	57 6	64 0	100
2000 γ " + 100 γ vitamin B_1	54 8	62 7	
0.5 cc. supernatant from inactivated pea root carboxylase			69.4

(*c*) vitamin B_1 pyrophosphate extracted by boiling for 3 minutes on a water bath, (*d*) total vitamin B_1 remaining in the denatured proteins following this extraction. The remaining portion was reprecipitated once and partially inactivated with pyruvate, as described previously. This fraction, known as Fraction II, was again precipitated and assayed in the same ways as Fraction I. The supernatants resulting from the reprecipitations of Fraction II were mixed together to give Fraction III. Fraction III was analyzed for total vitamin B_1 and for vitamin B_1 pyrophosphate as a check on the preceding assays. The results of this experiment are presented in Table III. The carboxylase activity measurements show that the pyruvate treatment caused inactivation of

the preparation to the extent of 51 per cent. This was completely reversed on addition of pyrophosphate, showing that no appreciable denaturation of the enzyme occurred. That reactivation with pyrophosphate is possible in spite of the removal of the greater part of the vitamin B_1 (Table III, 5th line) may indicate that this part of the vitamin B_1 is free; *i.e.*, not combined as cocarboxylase. This view is supported by the failure to find vitamin B_1 pyrophosphate in the supernatants (Fraction III). The portion of the vitamin B_1 which is not removed by the pyruvate treatment, but which remains in the proteins, is so strongly held

TABLE III

Fate of Vitamin B_1* and Vitamin B_1 Pyrophosphate during Treatment of Pea Root Carboxylase with Pyruvic Acid

The results are expressed per 100 mg. of the dry weight of the solids.

	Fraction I	Fraction II	Fraction III
Dry weight, *mg*	990	605	385
CO₂ per hr.			
No additions, *c.mm*	122	60	
104 γ vitamin B_1 pyrophosphate, *c.mm*	140	116	
2000 γ Na pyrophosphate, *c.mm*	151	151	
Total vitamin B_1, γ	1.02	0.545	1.45
Extractable vitamin B_1 pyrophosphate, γ	0.30	0.00	0.00
Total residual vitamin B_1, γ	0.42	0.49	

* The vitamin B_1 determinations by the *Phycomyces* method represent the total of the phosphorylated and unphosphorylated vitamin present in the sample.

that it is not extracted even after denaturation by boiling. This evidence, then, supports the hypothesis that pyruvate inactivation removes only pyrophosphate from the enzyme and that resynthesis occurs upon addition of the latter.

The fact that inactivated pea root enzyme preparations can be reactivated by pyrophosphate suggests that it might be possible to detect a significant cocarboxylase synthesis under these conditions. Experiments to test this possibility were carried out, but a synthesis could not be demonstrated with certainty. One negative result of particular interest follows. A pea root preparation was made and treated to remove pyrophosphate. It was

then pipetted into a series of Warburg vessels in the side arms of which had been placed previously water, cocarboxylase, and pyrophosphate solutions, respectively. The measurements were started and the contents of the side arms tipped in. When the next reading indicated an increased rate of CO_2 evolution (and, hence, presumably cocarboxylase synthesis) in the vessel containing pyrophosphate, the vessels were removed and immersed in boiling water. After cooling, 1 cc. of washed yeast was put into the side arm of each vessel and the vessels were replaced on the manometers. After equilibration the yeast was tipped in and the

TABLE IV

Attempt to Demonstrate Synthesis of Vitamin B_1 Pyrophosphate by Pea Root Preparation

Vessels 2, 3, and 4 contained, during the first run, partially inactivated pea root carboxylase, pyruvic acid, and the additions noted below. The contents of each vessel were then boiled, cooled, and 1 cc. of yeast added for the second run. Vessel 6 contained only vitamin B_1 pyrophosphate and pyruvate during the first run.

Vessel No.	Additions	CO_2 in 30 min., c.mm.	
		1st run	2nd run
2	Nothing	64.6	7.3
3	Vitamin B_1 pyrophosphate (10.4 γ)	74.0	23.6
4	Na pyrophosphate (2000 γ)	113.5	7.3
6	Vitamin B_1 pyrophosphate (10.4 γ)	0.0	171.3

cocarboxylase in each vessel was assayed. The results are shown in Table IV.

The failure to demonstrate a synthesis of cocarboxylase in the presence of pyrophosphate under the conditions of this experiment may be attributable to the fact noted earlier, that vitamin B_1 is held very firmly in the protein portion of the pea root carboxylase. Vitamin (and probably cocarboxylase) remains in the protein even after the latter has been denatured by boiling; as shown in the experiment of Table III, 90 per cent of the vitamin present in the partially inactivated preparation was retained by the denatured protein. It is possible that in the experiment of Table IV cocarboxylase was synthesized, but was not liberated by boiling.

The pea root preparation is capable of rapid destruction of

cocarboxylase. This is shown in Table IV by comparing the CO_2 production during the second run in Vessel 3 (pea root enzyme present) with that in Vessel 6 (pea root enzyme absent). This effect may be due to removal of cocarboxylase from the solution by the enzyme or to hydrolysis of the cocarboxylase into vitamin and pyrophosphate, followed by resynthesis from the liberated pyrophosphate and the vitamin bound in the protein.

DISCUSSION

It is shown in the experiments reported above that pea roots contain a carboxylase capable of decarboxylating pyruvic acid. The enzyme preparation contains appreciable amounts of vitamin B_1 and vitamin B_1 pyrophosphate. Treatments designed to dissociate vitamin B_1 pyrophosphate from the preparation reversibly were, however, unsuccessful, as described above. In these experiments it was found that by allowing the enzyme to act on pyruvic acid it could be partially inactivated, and that reactivation could be accomplished by the addition of either vitamin B_1 pyrophosphate or sodium pyrophosphate. It seems highly probable, from analogy with yeast carboxylase, that pea root carboxylase contains vitamin B_1 pyrophosphate as the prosthetic group. On this assumption, the evidence suggests that pyruvate inactivation is due to the splitting of pyrophosphate from the cocarboxylase molecule, and that resynthesis occurs upon addition of large amounts of pyrophosphate to the preparation.

A possible interpretation of the pyruvate inactivation is that a phosphorylation is linked with decarboxylation in the pea root enzyme (compare Lipmann (1939)). Attention should be called to the work of Peters and Sinclair (1933) which showed that addition of sodium pyrophosphate promotes oxidative decarboxylation by brain tissues.

SUMMARY

1. A protein preparation from pea roots contains a carboxylase capable of decarboxylating pyruvic acid in the presence of Mg^{++}.

Heat denaturation of the preparation liberates cocarboxylase (vitamin B_1 pyrophosphate).

2. The pea root enzyme is partially inactivated by allowing the preparation to act on pyruvate. Vitamin B_1, but not cocarboxylase, is found in the supernatant following precipitation of the pyruvate-inactivated enzyme. Heat denaturation fails to liberate cocarboxylase from the pyruvate-inactivated enzyme.

3. Reactivation of partially inactivated enzyme is accomplished by the addition of cocarboxylase, pyrophosphate, or the concentrated supernatant from the pyruvate inactivation.

4. The reactivation by pyrophosphate suggests that the essential feature of pyruvate inactivation is the removal of inorganic pyrophosphate from the enzyme.

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LIPIDS AND POLYSACCHARIDES IN ELECTROPHORET-
ICALLY SEPARATED BLOOD SERUM PROTEINS

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The occurrence of lipids in serum proteins separated by the aid
of electrophoresis has earlier been investigated by Bennhold (1)
and by Mellander (2). Bennhold used the electrophoresis ap-
paratus of Michaelis. The dye Sudan III was added to the serum
in order to make the lipids visible. It was stated that the Sudan
III boundaries coincided with those of the globulin in the electro-
phoresis experiments. Moreover, a positive Liebermann-Bur-
chard reaction for cholesterol was obtained in the globulin zone,
whereas this test was negative in the pure albumin zone. Benn-
hold concludes that the cholesterol of serum is bound to the globu-
lin and not to the albumin. He remarks, however, that on the
positive side often a non-colored globulin zone appeared that did
not contain cholesterol.

Mellander arrived at somewhat different results. He used
Theorell's apparatus. At the end of the electrophoresis experi-
ments the contents of the different cells of the U-tube were
analyzed quantitatively for cholesterol. On the anodic side
small amounts of cholesterol were found to migrate in front of the
albumin. In the cells containing only albumin cholesterol was
found too. In two instances cholesterol-free globulin was found
on the cathodic side. The albumin to cholesterol and globulin
to cholesterol ratios varied considerably in the different cells.
Mellander arrived at the conclusion that the cholesterol, if at all
bound to protein, probably is bound to subfractions of albumin
and globulin. The discrepancy between the results of these
authors may most easily be explained on the assumption that
the test for cholesterol used by Bennhold was not sensitive enough
to detect this substance in the separated albumin.

With regard to the phosphatides Bennhold states that he has not been able to find any regular relation between them and the electrophoretically separated proteins. The question of the relation between lipids and proteins in serum has often been studied by means of various other methods. Widely varying results have been obtained (see Sörensen (3), Theorell (4), Gardner and Gainsborough (5), Macheboeuf (6), Wolf and Frankenthal (7), and Handovsky (8)).

With the aid of the electrophoresis apparatus constructed by one of the authors (9) serum has recently been found to contain four electrophoretically well defined protein components; viz., albumin and three globulins, denoted as α-, β-, and γ- respectively. With the aid of this apparatus we have reinvestigated the problem just mentioned and also the distribution of carbohydrate in the different protein fractions obtained. One of the results obtained, namely the comparatively high lipid content of β-globulin, has already been referred to in a previous publication (10).

EXPERIMENTAL

The sera investigated were taken from three normal subjects and from a patient suffering from lobar pneumonia. The blood was drawn in the postabsorptive period.

The electrophoretic fractionation of the sera was performed in the large type of electrophoresis apparatus constructed by one of the authors. A brief description has been given elsewhere (10, 11). The cross-sectional area (7.5 sq. cm.) is 10 times larger than in the normal sized tube, and the capacity of each compartment is 35 ml. A special arrangement allows the U-tube to be built up of two, three, or four cells on top of each other. The bottom cell, which is of a construction similar to that of the normal size, is usually filled with glass beads to minimize the dead space volume that otherwise would be considerable in this construction. As the cross-sectional area of the electrophoresis tube is so much larger, it is impossible to use high potential gradients. In order to make use of the separation capacity of the apparatus to its full extent one has to increase the time for an experiment to 1 day or more.

The size of the electrodes and the electrode vessel volumes have also been increased in accordance with the increased volume of

separation (see (9)); each electrode is made of about 500 gm. of silver wire and the capacity of each electrode vessel is 4.5 liters. Only the central U-tube part is enclosed in the bath (which is kept a few tenths of a degree above zero), whereas the electrode vessels are mounted outside (see Fig. 1), thus avoiding the construction of a very large bath. For filling, emptying, and cleaning, the U-tube is detached from the electrode vessels, which are left mounted in their position and can be emptied and refilled by suction and syphon arrangements.
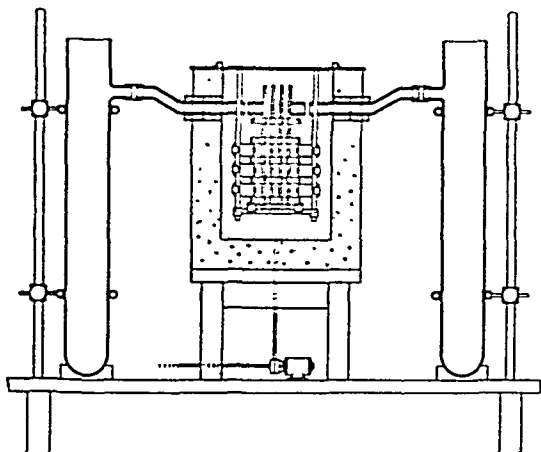
FIG. 1. Diagram of the electrophoresis apparatus

For optical observation the simple schlieren method is sufficient. The head lens should be about 200 mm. but may be of rather poor quality, as no measurement, only localization of the boundaries in connection with preparative work, is intended with this construction. It is of advantage to mount the lens as close as possible to the U-tube, or to use two lenses, one on each side, arranged to give parallel light through the bath.

In general the fractionations were carried out with undiluted sera dialyzed against a buffer solution containing 0.0098 M Na_2-HPO_4, 0.0008 M NaH_2PO_4, and 0.0700 M NaCl, which gives a pH of about 8 and an ionic strength of 0.1. A weaker buffer, though of advantage from the point of the highest possible voltage, is not

advisable on account of the boundary anomalies at the high protein concentrations in question.

During the larger part of an experiment "automatic" compensation was used. The hydrostatic pressure caused by the migration of the serum protein column is sufficient to give rise to a backward displacement of the whole column in the U-tube, if open electrode vessels with large, free surface areas of solution are used. Towards the end of the experiment, in order to move the boundaries to the position desired, a simple compensation was arranged with a capillary syphon.

For pipetting out the various fractions the convection-free pipette arrangement described earlier was used (10, 11). On the positive side three fractions were usually taken out; namely, (1) albumin, (2) albumin + α-globulin, (3) albumin + α-globulin + β-globulin. On the negative side also three fractions are obtained, (4) γ-globulin, (5) γ-globulin + β-globulin, (6) γ-globulin + β-globulin + α-globulin. Thus the separation gives at once pure albumin and γ-globulin. For obtaining α-globulin one must run a new separation (in the smaller apparatus) with Fraction 2 or 6; for obtaining β-globulin, with Fraction 3 or 5. The preparation of α- and β-globulins in this way did not, however, give satisfactory results. At the high protein concentrations necessary to give reasonably large amounts the boundary anomalies are very pronounced, which makes the boundaries on the descending side very diffuse. Therefore, comparatively large amounts of good purity are obtained without difficulty only of Fractions 2 and 3 but the further electrophoretic fractionation of these to obtain the slower globulins again necessitates taking samples from the descending column, where the separation is bad. These unfavorable circumstances led us to try another procedure earlier suggested by one of the authors (see (12)) for the isolation of the two intermediate globulins. By this method it is possible to obtain all the fractions without repetition of the fractionation. The current was allowed to go in one direction for about 70 hours. After the pure albumin and the γ-globulin layers are carefully pipetted off, so that sharp boundaries between buffer and albumin + α-globulin and between buffer and γ-globulin + β-globulin respectively remained as intact as possible, the current was sent through the remaining column in the reverse direction for about

35 hours or more. As is easily realized, the top layers now formed on the ascending and descending sides will consist of α-globulin and β-globulin respectively. After such a long run one cannot expect to get absolutely pure fractions even by this method. A final run in the smaller apparatus increased the purity and made possible a quantitative estimation of the impurities by registration of the optical concentration gradient by the method of crossed slits (Svensson (13)).

The electrophoretic preparation of the α- and β-globulins is therefore a rather difficult task with the present technique and it may very well be that simpler methods can be found; e.g., precipitation under suitable conditions. This question will be dealt with in another communication. For the purpose of the present publication it was considered essential to use only electrophoresis, as it seems to be the most gentle method available.

The chemical analyses of the protein fractions isolated were carried out as follows:

Cholesterol—The solutions were extracted according to the method of Bloor with 20 volumes of alcohol-ether, 3:1. After evaporation of the larger part of the ether, 0.1 ml. of 15 per cent NaOH was added and the evaporation continued until a few ml. only of the solution were left. The rest of the fluid was driven off in the boiling water bath with the aid of a stream of CO_2. The quite dry residue was thoroughly extracted with chloroform and determined colorimetrically according to ˙the method of Theorell. In the later experiments the method of Schoenheimer and Sperry was employed for the cholesterol analyses. With this method considerably smaller amounts of cholesterol can be determined than with Theorell's method.

The lipoid P was quantitatively estimated as follows: The protein solutions were extracted with 25 volumes of alcohol-ether, 2:1. The mixture was heated to boiling, cooled, and filtered. The precipitate was well washed with ether, the filtrate evaporated to dryness, and the phosphorus in the residue determined colorimetrically according to Theorell's modification of the Fiske-Subbarow method. Repeated control experiments showed that in the procedure described no trace of inorganic phosphorus was obtained.

For the determination of the carbohydrate components the

protein precipitates from the lipid extractions were evacuated free from ether and dried at +100° *in vacuo* over P_2O_5 until constant weight was attained. As these precipitates in addition to proteins contained variable quantities of the buffer salts used in the electrophoresis, their protein content must be determined by nitrogen analysis (micro-Kjeldahl). The method of Sörensen and Haugaard was used for the determination of the carbohydrate. This method cannot be considered very accurate. However, for the present purpose, its accuracy is no doubt quite sufficient. The results obtained were calculated as mannose. In the albumin, which could be obtained in considerably greater quantities than the globulins, glucosamine was determined according to the method of Elson and Morgan as modified by Nilsson (14).

In some instances the isolated albumin and γ-globulin were submitted to a second electrophoresis lasting 5 to 7 hours in the medium sized (10 ml.) apparatus. In these experiments the migration of the protein was overcompensated. In that way the upper end of the protein solution on the positive side was slowly driven out of that cell in which it was contained at the start. The current was switched off when the protein boundary on this side passed through the bottom of the cell mentioned. This procedure aimed at a separation of the protein from the free lipids eventually present. The cells emptied as described above regularly proved to contain a small amount of protein. Calculated in per cent of the protein present, the amount of lipid in these cells was regularly considerably greater than in the cells containing the main part of the protein. This probably indicates either that a part of the lipids had occurred in a free state before the second electrophoresis or that a certain dissociation between protein and lipid had taken place during this procedure.

As a rule only a minor part of the lipid present in the protein solution after the first electrophoresis could, in the way just described, be removed in the second one. Thus, in normal Serum II the cholesterol of the albumin in the course of the second electrophoresis decreased from 1.30 to 1.08 per cent. The lipid P at the same time was lowered to 0.10 per cent from 0.13 per cent.

In the albumin of Serum III the cholesterol of the albumin similarly decreased from 0.92 to 0.84 per cent. The change in

the phospholipid did not in this case exceed the experimental error.

As seen from Table I, all protein fractions of serum contain lipids, although in varying amounts, γ-globulin being poorest. Yet the quantities of cholesterol and phospholipid associated with the γ-globulin are such that, assuming a molecular weight of this protein of 150,000, the protein should in all cases but one bind 1 or more molecules of lipid per molecule (1 molecule of lipid per molecule of γ-globulin should give 0.25 per cent cholesterol and about 0.5 per cent phospholipid). The lipids of the γ-globulin might also be bound to a subfraction of this protein, the rest being unasso-

TABLE I

Lipids of Various Protein Fractions of Serum
The results are given in per cent.

Serum No.	Albumin		α-Globulin		β-Globulin		γ-Globulin	
	Cho-les-terol	Lipoid P	Cho-les-terol	Lipoid P	Cho-les-terol	Lipoid P	Cho-les-terol	Lipoid P
I. Normal...... .	0.99	0.06			4.6	0.31		0.05
II. "	1.30	0.13		0.30		0.44	0.71	0.03
III. "	0.92	0.08	4.45	0.28	12.7	0.46	0.11	0.03
Mean......... .	1 07	0.09	4.45	0.29	8.65	0.40	0.41	0.04
Calculated as phospholipid		2.25		7.25		10.0		1.0
Pneumonia...............	0.71	0.02	0.42	0.21		0.56	0.28	0.12

ciated with lipids. This possibility is somewhat supported by the rather great variations of the cholesterol values of the γ-globulin. It is also a general experience from electrophoresis work on sera that γ-globulin shows the greatest inhomogeneity of the protein fractions. In the albumin too the lipid values are relatively low. The cholesterol values are definitely higher than those of the γ-globulin. It is noteworthy that these cholesterol values agree rather well with that calculated for a compound of 2 molecules of the lipid and 1 molecule of albumin (mol. wt. 68,000; calculated 1.14 per cent cholesterol) even if this may be due to pure chance.

The lipid content of α- and β-globulins is considerably greater.

The phosphatides as calculated from the lipoid P values amount to 5 to 7.5 per cent of the α-globulin and 8 to 14 per cent of the β-globulin.[1] Owing to the great difficulty of obtaining pure α-globulin in sufficiently large amounts, cholesterol could be determined only once in α-globulin from normal serum. In the pneumonia serum a consistently lower cholesterol value was obtained for the α-globulin, this being the only instance in which the pneumonia serum showed a marked difference from the normal.

Even in serum of blood drawn from subjects fasting in the morning the β-globulin, as it appears in the electrophoresis apparatus, exhibits a more or less pronounced opalescence. The boundaries of this opalescence coincide exactly with those of the β-globulin. The opalescence in all probability is due to suspended lipid particles. Migrating with the same velocity as the β-globulin, these particles no doubt are coated with a protein film which determines their electrophoretic behavior. In the other protein fractions, including α-globulin, the lipid appears to be more highly dispersed.

The results given here are on the whole in good agreement with those of Mellander and support the assumption made above that the test used by Bennhold for the detection of cholesterol has not been sensitive enough to indicate the cholesterol in the albumin fraction. When Bennhold found the cathodic end of the globulin column to be free from cholesterol, he had obviously tested a solution containing γ-globulin only.

The result of the analyses performed on proteins after a second electrophoresis makes it quite clear that the lipids present in the solution of the electrolytically separated proteins are not free but to a large extent bound to protein in an unknown manner.

The outcome of the carbohydrate analyses (Table II) is in agreement with earlier findings in so far as the globulins are found to contain more carbohydrate than the albumin. α- and β-globulins are the protein fractions richest in carbohydrate. Calculated as dihexosamine, the carbohydrate content of the α- and β-globulins is 9 to 12 per cent. The sum of the lipids and carbohydrates in the β-globulin amounts to as much as 20 to 30 per cent of the protein.

[1] It is of interest to note that Longsworth, Shedlovsky, and MacInnes (15) in their electrophoretic analysis of pathological sera found that a considerable part of the abnormally large β component in plasma from a patient with obstructive jaundice could be extracted with ether.

It should be remembered that the α- and β-globulins, although very rich in lipids and carbohydrates, constitute only a minor part of the total serum proteins. Consequently as much as half or more of the total lipids and total carbohydrates of the serum is bound to the albumin and the γ-globulin, in spite of the low percentage composition.

The serum albumin from a patient with pneumonia contains considerably more carbohydrate than the albumin of normal sera. This agrees with earlier observations by Nilsson (14).

In some of the prolonged separations the albumin boundary split up into two but the difference in mobility was very small (only about 2 per cent of the total mobility). A separation was nevertheless tried in one case (a pneumonia serum), which, however, was not complete. The faster component was colorless; the

TABLE II

Per Cent of Carbohydrate in Protein Fractions of Serum

Serum No.	Albumin		α-Globulin, mannose	β-Globulin, mannose	γ-Globulin, mannose
	Mannose	Glucosamine			
II. Normal..................	1.1	0.45		8.3	3.6
III. "	1.2	0.56	6.0	6.2	3.0
Pneumonia..................	5.8	3.6	9.9	1.5	3.7

slower showed the yellow (bilirubin) color which usually migrates with the albumin. Analysis showed that the faster fraction contained 4.6 per cent carbohydrate (calculated as mannose) and the slower only 2.0 per cent.[2] As the fractions were far from pure, these figures only indicate that there is a difference in the composition of the fractions but do not give the carbohydrate content of the pure components. Therefore the relation between these and those obtained by Hewitt (16) and by Kekwick (17) cannot yet be definitely established. Recently Luetscher (18) has shown that carbohydrate-free serum albumin may be fractionated by electrophoresis at a more acid pH into two components with different mobilities.

[2] Owing to the small amounts available, the correction for buffer substances could not be made in the analysis of these samples. The values are therefore approximate only.

SUMMARY

With the electrophoresis technique of Tiselius the question of the relations between the serum proteins on the one hand and that of the lipids and the polysaccharides of serum on the other were investigated.

The electrophoretically separated proteins, albumin and α-, β-, and γ-globulins, all contained cholesterol and phospholipids. The α- and β-globulins were, however, far richer in these lipids than the two other proteins mentioned. In the β-globulin layer part of the lipids appear to exist in a more coarsely dispersed form than in the other serum proteins.

All serum proteins which could be separated with the aid of electrophoresis likewise contained carbohydrates. Also here the α- and β-globulins showed the highest percentage.

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ELECTROPHORESIS OF LIPID-FREE BLOOD SERUM

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The finding of lipid substances in all electrophoretically separable serum proteins, in the case of α- and β-globulins in remarkably large proportions (Blix, Tiselius, and Svensson, 1941), raised the question of the possible rôle of the lipids in the electrophoretic behavior of these proteins. Experiments with sera from which the lipids had been largely removed, according to the mild extraction principle introduced by Hardy and Gardiner (1910), seemed likely to give certain information in this respect. Such experiments were accordingly carried out. At the same time some questions pertaining to the transport function of the serum proteins were taken into consideration and experimentally investigated.

EXPERIMENTAL

The serum lipids were removed in the following way. 10 ml. of serum, cooled to $\pm 0°$, were precipitated with 60 ml. of acetone at $-14°$ and immediately centrifuged for 3 minutes at 3000 R.P.M. (the centrifuge was kept at a temperature not exceeding $+10°$). The supernatant fluid was poured off and the precipitate treated for $\frac{1}{2}$ hour with 60 ml. of fresh acetone of $-14°$. The mixture was placed in a refrigerator adjusted to this temperature. This treatment was repeated once. The precipitate was then well stirred up with 60 ml. of a mixture of acetone and dry ether, 2:1, allowed to stand for 5 minutes, and again centrifuged. The precipitate was at last treated with 60 ml. of dry ether for 15 minutes, this treatment being repeated once. The treatments with acetone-ether and ether were also carried out at $-14°$. The precipitate was then placed in a vacuum desiccator and freed from ether by

evacuation with an oil pump at ordinary room temperature. During the whole procedure great care was taken not to expose the precipitate for more than minimal periods of time to the moisture of the air. Subsequent extraction of the precipitate with dry ether in a Soxhlet apparatus was not carried out, as it did not appear that further lipids would be removed. In fact not more than traces of lipids were extracted after the two first acetone treatments, the subsequent treatments with ether serving mainly to remove the acetone.

With the extraction procedure thus employed the serum cholesterol was completely removed. On the other hand a certain amount of phospholipids (about 25 per cent of the total) was not extracted. An analysis according to the procedure of Brante (1940) showed the unextracted phospholipid to be mainly cephalin, whereas the phospholipid in the extract was chiefly lecithin. The proteins after the extraction dissolved easily and completely in the buffers used.

The electrophoresis apparatus of Tiselius (1937, *a*) was employed. All measurements were made at $\pm 0°$ with a potential gradient of about 5 volts per cm. The sera were diluted twice with and dialyzed against phosphate buffers of constant ionic strength, $\mu = 0.1$.

Table I shows the mobility values of the serum proteins found in native and lipid-free sera at different pH values. Sera I to IV were drawn from healthy men in the postabsorptive period. Serum V was a horse serum.

In the experiments with lipid-free sera the β-globulin, contrary to observations on native sera, always appears quite clear. This confirms the surmise that the opalescence of the β-globulin in native sera is due to the lipid substances present.

It may be gathered from Table I that no α-globulin appears in lipid-free sera at pH 6.1 and 7.4. In four further experiments performed at these pH values with sera to which certain foreign substances had for other purposes been added (see below) no α-albumin boundaries appeared either. The horse serum, in which the α-globulin is quantitatively more important than in human serum, agreed with the latter in this respect. On the contrary at pH 8 the α-globulin boundaries in most cases became visible also in the lipid-free sera, although—especially in horse serum—the

schlieren band was much less marked than in normal serum. In two experiments at pH 8, with foreign substances added to lipid-free sera, the α-globulin likewise appeared.

The changes affecting the α-globulin represent the only difference in electrophoresis found between the native and the lipid-free sera.[1]

Whether the effect on the α-globulin is due to the absence of the lipids or to an incipient denaturation of the protein cannot with certainty be decided. The easiest explanation is perhaps that the mobility of the α-globulin, due to an incipient denatura-

TABLE I

Electrophoretic Mobilities of Serum Proteins

The results are given in cm.^{2} sec.$^{-1}$ volt^{-1} $\times$ 10^5.

Serum No.	pH	Kind of serum	Albumin	α-Globulin	β-Globulin	γ-Globulin
III	6.1	Native	−3.8	−3.0	−1.9	+0.3
"	6.1	Lipid-free	−3.8		−1.9	+0.3
I	7.4	Native	−6.6	−5.2	−3.8	±0.0
"	7.4	Lipid-free	−6.5		−3.8	±0.0
II	7.4	Native	−6.5	−5.2	−3.7	±0.0
"	7.4	Lipid-free	−6.5		−3.8	±0.0
V	7.4	Native	−6.2	−5.0	−3.7	−0.4
"	7.4	Lipid-free	−6.2		−3.7	−0.3
III	8.0	Native	−7.5	−6.2	−4.7	−0.4
"	8.0	Lipid-free	−7.5		−4.7	−0.4
IV	8.0	Native	−7.5	−6.5	−4.3	−0.5
"	8.0	Lipid-free	−7.7		−4.7	−0.3
V	8.0	"	−7.5	−6.2	−4.5	−0.9

tion, suffers such a change that its boundaries submerge in the broad schlieren band of the albumin or the β-globulin, the possible difference in mobility between one of these proteins and the changed α-globulin being so small that even in extended experiments no separate α-globulin boundaries become visible. The

[1] Tiselius (1937, *b*) mentions an experiment with serum from which the lipids had been removed in much the same way as in the present work. A large reduction of the mobility of the albumin was the sole change noted. Possibly the precautions taken in order to prevent protein denaturation were not in this case so strict as in the present experiments.

facts that the α-globulin appearing at pH 8 in lipid-free sera shows a quite normal mobility and that the mobility of the other protein fractions is, within the experimental error, unchanged nevertheless contradict such an explanation. That the α-globulin, deprived of its lipids, assumes as such a mobility coinciding with one of the other proteins or even that the native α-globulin has the nature of a compound with one of the other proteins with lipids seems also to be disproved by the appearance of α-globulin at pH 8. Lastly, it is perhaps conceivable that the lipid-free α-globulin particles become coated by one of the other lipid-free serum proteins, say the β-globulin, the resulting complexes having the mobility of the latter and not being stable on the alkaline side of about pH 7.5. The knowledge gained in the experiments given below might to some extent support this view.

A series of electrophoresis experiments was carried out with lipid-free (and native) sera to which had been added suspensions of either cholesterol, mastic, or the dye Sudan III.

The cholesterol and the mastic were dissolved in warm alcohol and poured into boiling water. The alcohol was driven off by concentration of the solution to one-third of the original volume. The cholesterol suspension was stabilized by addition of a trace of sodium oleate. The sols obtained contained about 1 per cent of the substance suspended. The Sudan III sol was prepared according to the method of Bennhold (1932). Of these suspensions usually 0.5 ml. was added to a mixture of 4 ml. of serum and 7.5 ml. of buffer solution.

In lipid-free sera the opalescence of the mastic and cholesterol respectively at pH 6.1 and 7.4 always coincided exactly with the β-globulin boundaries. At pH 8 the opalescence boundaries appeared between the α and β boundaries, save for one instance when they coincided with the α-globulin boundaries.

The results of the experiments with Sudan III are given in Table II.

From these results it is clear that the use of Sudan III as an indicator of the lipid substances of serum in electrophoresis experiments (Bennhold, 1932) is not justified. There is obviously reason to be cautious with the use of Sudan III as an indicator of lipid substances. This is further evidenced by the observation that in a native serum from a patient with marked alimentary

lipemia the opalescence boundaries migrated between the boundaries of the α- and β-globulins, whereas the Sudan III at the same time migrated exactly with the β-globulin. It should be added here that in two further sera from individuals with marked alimentary lipemia the opalescence boundaries likewise appeared between the α- and β-globulin boundaries.

In this connection another observation made in the above experiments should be pointed out. The albumin, which on electrophoresis of native sera always appears yellow, owing to the adhering bilirubin, in the lipid-free sera regularly appears colorless, or, in some instances, only faintly yellowish. As the bilirubin does not go into the extracts, it evidently shifts over to the only protein appearing colored in these sera; namely, the β-globulin.

TABLE II

Use of Sudan III As Indicator of Lipid Substances of Serum

Serum No.	pH	Kind of serum	Sudan III migration
II	7.4	Native	With β-globulin
"	7.4	Lipid-free	" "
IV	7.4	Native	Between α- and β-globulin
"	8.0	"	" " " "
V	8.0	Lipid-free	With β-globulin

From the observations thus made it appears that the β-globulin plays a special rôle in the transport of substances differing in chemical structure but similar in that they may occur in serum as hydrophobic colloidal particles.[2] As shown by Blix, Tiselius, and Svensson (1941), all the electrophoretically separable serum proteins contain lipids. However, whereas the lipids of albumin, α-globulin, and γ-globulin respectively probably should be regarded as relatively permanent elements in these proteins, it is suggested that the β-globulin lipids, which, to a great extent at

[2] The underlying observations may appear to be somewhat at variance with the results of Moyer and Moyer (1940) who worked on electrophoresis of quartz and collodion particles in serum and with those of Ludlum, Taft, and Nugent (1931), who investigated the electrophoresis of chylomicrons. The discrepancies are, however, not so great that they may not be in the main explained by the differences in the experimental conditions employed.

least, are present as comparatively large particles coated by a protein film, are lipids passing through the blood on their way from one part of the body to another. The β-globulin is thus supposed to be endowed with a special function in the fat transport of the body. In marked lipemic conditions the turbidity, as mentioned above, often appears between the α- and β-globulin boundaries. This probably indicates that, when larger amounts of fat have to be transported, the albumin or the α-globulin or both of them in addition to the β-globulin go into the surface film of the lipid particles and thus, in an auxiliary way, take part in the fat transport. The outcome of the experiments with mastic and cholesterol at pH 8 might indicate that a participation of proteins with a greater mobility than the β-globulin in the trans port of materials of the kind here in question takes place, especially at more alkaline pH values.

The substances which are transported with the different serum proteins may undoubtedly influence the transport conditions of each other. Competition for the transporting proteins and displacements certainly may occur. The distribution of the transported substances on the different protein components does not to all appearances represent a fixed state but rather a labile equilibrium which is often and easily changed in the one or the other direction. Such changes might have far reaching consequences, especially in pathological conditions.

SUMMARY

In sera from which the lipids had been largely removed by precipitation and extraction with organic solvents at low temperature the migration velocities of the different protein components were found to be unchanged, except that the α-globulin boundaries regularly disappeared at pH values below 8. The cause of this change is discussed.

Cholesterol, mastic, and the dye Sudan III, which were added to lipid-free sera in the form of suspensions in water, as a rule migrated with the β-globulin. In lipid-free sera the bilirubin is completely, or for the greater part, shifted over from the albumin to the β-globulin.

Certain aspects of the transport function of the serum proteins are discussed.

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X-RAY STUDIES ON TUBERCULIN PROTEINS[*]

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The molecular structure of non-amorphous organic substances, as found from study of their x-ray diffraction patterns, is discovered to best advantage when the substances are chemically pure. Purity of compounds is not yet strictly fulfilled in most biologically active materials. However, in the tuberculin proteins prepared and studied by Seibert and coworkers (1–4), which we have used, we have substances which have been chemically analyzed with precision. This has given us the chance, in our experiments with x-ray analysis of tuberculin proteins, to study the influence of concomitant substances upon the x-ray diffraction patterns of the former. By comparison and elimination the x-ray diffraction pattern of reasonably pure tuberculin proteins can thus be established. Finally determinations of the particle size according to Scherrer's (5) method were made on three samples of tuberculin proteins for which ultracentrifugal evaluations of the molecular weight are available (3).

Preparations Studied

Several methods of isolating the active principle from tuberculin have been used. In general the main difference was that some fractions, such as the TPU-H2 and TPA-1, were made from unheated tubercle bacillus culture filtrates (1) and were antigenic, while the remaining fractions were isolated after the cultures had been heated, and were thus non-antigenic. TPU-H2 was

obtained by concentrating raw culture filtrate by ultrafiltration and washing with 0.5 per cent phenol at room temperature. It was then dried in the desiccator.

TPA-1 was prepared by repeated half saturation with ammonium sulfate at pH 4.8 and then dialysis against water and drying in the desiccator.

PPD-98970, PPD-Rx, and SOTT-19 were made in three different laboratories according to the standard procedure (2) previously used for preparing PPD, which involved precipitation with trichloroacetic acid.

SOTT-b2 and SOTT-b3 were separated from SOTT-19 and further purified as previously described (3).

PPD-62 (avian), PPD-61 (bovine), and PPD-65 (human) were made by repeated precipitation with ammonium sulfate (half saturation) and then 2 per cent trichloroacetic acid. The concentration by ultrafiltration was done at 5–6°, no preservative was used, and the final product was dried in the frozen state. PPD-67 (human) was treated similarly, except that it was separated by precipitation at pH 4.3.

PPD-49608 and PPD-49609 were made by the latest procedure (4), involving precipitation at pH 7.0 and giving the least denaturation possible. Thus these products have also some of the lowest contents of impurities.

The tuberculin polysaccharide was isolated from tuberculin filtrate (3), and contained only 0.097 per cent nitrogen.

The tuberculin proteins investigated and their content of buffer substances, nucleic acid, and polysaccharide, as well as determinations of the molecular weight, are summarized in Table I. The radius of the diffraction rings and a characterization of the type of the corresponding photometric graphs are included.

Apparatus and Technique—We used for these experiments an x-ray apparatus constructed by two of the authors, the details of which have been reported earlier (6). The tube operates at a peak voltage of 35 kilovolts, at an average current of 15 milliamperes. A copper target, aluminum foil window (0.013 mm.), and a lead diaphragm (1 mm. in diameter and 47 mm. in effective length) produce a nearly monochromatic[1] x-ray beam of 1.54 Å.

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[1] We ascertained in special experiments that use of K_α radiation alone (nickel filter of 0.002 cm. thickness) does not appreciably modify the diameters in our particular diffraction patterns.

TABLE I

Comparative Data from X-Ray Diffraction, Sedimentation, and Analyses

Tuberculin protein fraction	Buffer	Per cent nucleic acid	Per cent polysaccharide	$S_{20} \times 10^{-13}$	Mol. wt.	Type of diffraction pattern	Diffraction pattern, mm. Radius of larger ring	Radius of smaller ring
PPD-67, human	5% protein in phosphate buffer, pH 7.3	3.0	6.0			Serum albumin	13.6	6.1
PPD-62, avian	1.5% protein in phosphate buffer, pH 7.3	1.2	4.1			" + salt indentation	13.0	
TPA-1	Dialyzed against water	0.3	6.8	3.3 1.5		Serum albumin	13.8	6.1
PPD-65	1.7% protein in phosphate buffer, pH 7.3	0.8	4.8			" + faint salt indentation	13.3	5.8
PPD-Rx	Free of excess salts	7.2	5.1			Serum albumin	13.2	5.5
PPD-61, bovine	1.3% protein in phosphate buffer, pH 7.3	1.8	4.4	1.4	15,700*	" + faint salt indentation	12.9	5.5
TPU-II2	Dialyzed against water	5.6	66.5	1.8†		Polysaccharide	13.0	
PPD-98970	Free of excess salts	17.0	25.6			"	13.0	
SOT'l-19	" " " "	21.8	24.0	1.1†		Nucleic acid	13.2	5.9
SOT'l-b3	" " " "	3.0	4.4	1.2	17,000	Serum albumin	13.2	5.7
SOT'l-b2	" " " "	5.7	9.6	1.5†		"	13.6	5.8
PPD-49609	Dialyzed against water	1.7	5.5	1.3†	14,500‡	"	13.8–13.9	5.9
PPD-49608	" "	1.2	7.0			"		

* The sedimentation and diffusion constants of this preparation were made by Professor A. Tiselius and coworkers at the University of Upsala, Sweden.

† Heterogeneous.

‡ The sedimentation and diffusion constants of this preparation were made by Mr. Dennis W. Watson in the laboratory of Professor J. W. Williams at the University of Wisconsin.

The specimen, pressed into a hole drilled into a microscopic glass slide 1.32 mm. in thickness, is 88 mm. from the focal spot and 37.7 mm. from the film. An exposure time of 1 hour is adequate for most substances.

In order to obtain an objective evaluation of the x-ray diffraction patterns, a relative light transmission graph[2] of each film was made with the help of a photoelectric microdensitometer (Spiegel-Adolf and Peckham (7)). By using the method developed in this paper an exact center determination, and therefore an exact determination of the radius of the diffraction rings, is made possible. The same apparatus and a similar method were used for the photoelectric determination of the density graphs (density $= \log t_0/t_1$).

Results

The transmission graphs of the x-ray diffraction patterns of the thirteen tuberculin protein preparations are not identical and can be divided into at least four groups.

Group 1—Group 1 consists of three tuberculin proteins, PPD-62, PPD-65, and PPD-61. Of these only the first one shows a really distinctive diffraction pattern. This pattern contains at least two more rings than any of the other tuberculin proteins. These rings have radii of 16 and 24 mm. respectively. Both rings are identical with rings occurring in the diffraction pattern of the phosphate buffer which was used up to a concentration of 14 per cent in the preparation of PPD-62. Photometric graphs of the tuberculin protein and of the buffer substance reveal more details (Fig. 1). The influence of these findings upon the explanation of the x-ray diffraction pattern of PPD-62 (avian) seems obvious. PPD-61 and PPD-65 show only very slight inflections at 16 mm. from the center. We ascertained in further experiments that phosphate buffer admixture up to 5 per cent did not influence the shape of the x-ray diffraction patterns made with our equipment

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[2] The relative transmission of an x-ray diffraction film is the ratio between the light transmission at the point under observation and the light transmission of the center of the film, which has been protected against x-rays by a plate of lead but otherwise undergone the same treatment as the rest of the film (t_1/t_0). For our graphs the relative light transmission is plotted against the distance in mm. from the center of the x-ray diffraction pattern.

and technique. As a precaution, however, dialysis against distilled water was thereafter used in order to rid the tuberculin proteins of excessive salt.

Group 2—The light transmission graph of one sample, tuberculin TPU-H2, is different from those of the other samples in that the graph shows a wider and deeper minimum, which is closer to the center than the second minimum in most of the other samples. Tuberculin TPU-H2 contains 66.5 per cent of polysaccharide. A corresponding analysis of pure tuberculin polysaccharide reveals
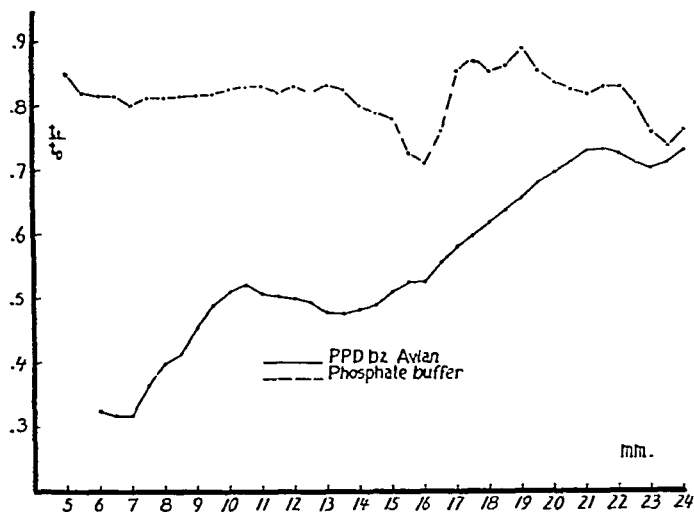
FIG. 1. Photometric transmission graphs of PPD-62 (avian) (unbroken line), original solution containing 1.5 per cent protein in phosphate buffer, and phosphate buffer (broken line). t_1/t_0 indicates relative transmission; mm. indicates distance from the center of the x-ray diffraction pattern.

a great similarity in its diffraction pattern, indicating that it is probably responsible for the particular shape of the diffraction pattern of tuberculin TPU-H2 (Fig. 2). 4 to 7 per cent of polysaccharide in the tuberculin proteins seems not to modify appreciably their x-ray diffraction patterns.

Group 3—Two transmission graphs, *viz.* of tuberculin proteins PPD-98970 and SOTT-19, are similar to each other and different from the rest. The difference consists in the fact that the transmission graphs show only a gentle slope and very little of the two

dips observed in the rest of the graphs, which will be described in the next paragraph. Both tuberculin proteins PPD-98970 and SOTT-19 contain 17 or more per cent nucleic acid (see (8)). Therefore x-ray diffraction patterns of a very pure preparation of thymus nucleic acid[3] were made. The corresponding transmission graphs at distances of 4 to 16 mm. from the center are horizontal lines. Although the presence of nucleic acid could thus be used to explain the behavior of the x-ray diffraction pattern of the two tuberculin proteins in the same way as we have explained the influence of salt and polysaccharide, a more exact proof is avail-
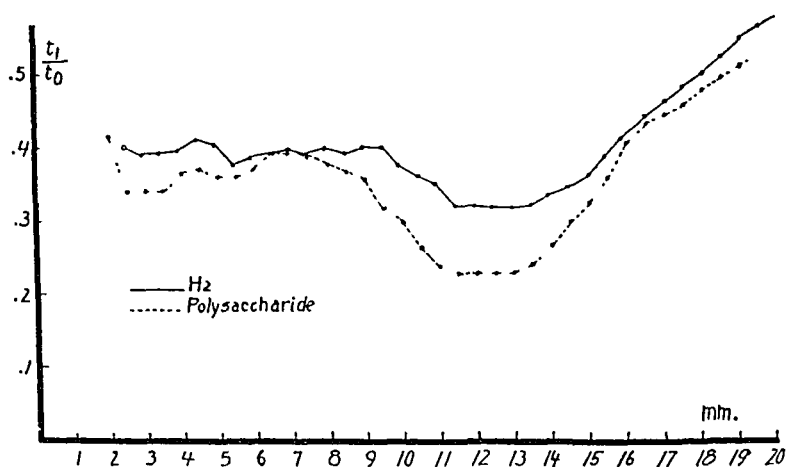
FIG. 2. Photometric transmission graphs of TPU-H2 (unbroken line), containing 66.5 per cent polysaccharide, and tuberculin polysaccharide (broken line). t_1/t_0 indicates relative transmission; mm. indicates distance from the center of the x-ray diffraction pattern.

able. SOTT-19 was almost freed of the concomitant nucleic acid, resulting in a preparation referred to as SOTT-b3. When the x-ray diffraction pattern of the latter is studied with our photoelectric microdensitometer, the transmission graph shows that our explanation that the nucleic acid causes a flattening out of the graph was correct. SOTT-b3 shows transmission graphs very similar to the graphs described in Group 4 (Fig. 3).

The results of these studies suggest the use of x-ray diffraction methods for analytical purposes in the study of contaminations in biological substances.

[3] This preparation was given one of us by Professor Einar Hammarsten.

Group 4—Nine samples out of thirteen tuberculin proteins show similar x-ray diffraction patterns. They are characterized by two distinct diffraction rings, corresponding to spacings of 4.43 to 4.60 Å. and 9.64 to 10.6 Å.[4] These spacings are almost identical with the ones reported by Astbury and Lomax (9) for the various undenatured and denatured proteins. The average of the spacings given above also corresponds very closely to the values 4.5 and 10.2 Å. found by Katz (11) and coworkers for crystallized proteins. The fact that the backbone spacing is more constant (variations of ± 0.1 Å. about its mean of 4.5 Å.) than the side
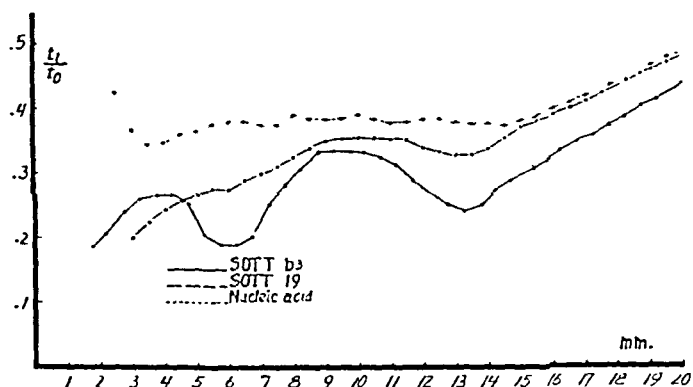
Fig. 3. Photometric transmission graphs of SOTT-b3, containing 3.0 per cent of nucleic acid (unbroken line), SOTT-19, containing 21.8 per cent of nucleic acid (broken line), and nucleic acid (dotted line). t_i/t_0 indicates relative transmission; mm. indicates distance from the center of the x-ray diffraction pattern.

chain spacing (variations of more than 0.5 Å. about its mean of 10.26 Å.) has been reported by Astbury and Lomax (9) for proteins.

[4] In the above computations we have followed the example given by other authors (Astbury and Lomax (9), Clark and Shenk (10), Katz (11)) in the use of the Bragg equation for the numerical interpretation of the x-ray diffraction patterns of proteins. Nevertheless there may be possible objections to the use of the Bragg equation in these cases. It has been called to our attention that our results in the computation of the particle sizes according to the method of Scherrer would only yield two layers of the 10 Å. kind and only about five layers of the 4.5 Å. kind in the average particle, and these numbers of layers are much smaller than those contemplated in the derivation of the Bragg equation.

The rather wide range of the spacings for an individual protein may be explained by the presence of varying amounts of concomitant substances (up to 7 per cent polysaccharides and up to 5 per cent nucleic acid).

Besides, there may be still other reasons for differences in the diffraction patterns. Four of nine tuberculin proteins, PPD-Rx, PPD-61 (bovine), SOTT-b3, and SOTT-b2, show in varying degree the characteristics of denaturation as described by Astbury and Lomax (9) and confirmed by our own findings with heat-denatured proteins (12). The main characteristics consist not only in a sharpening of the backbone reflection, which, instead of a diffuse ring, appears as a sharp line, but also in small changes of the spacings of both reflections. In our group of five apparently undenatured tuberculin proteins the average spacings were 4.48 Å. (4.43 to 4.57) and 9.87 Å. (9.64 to 10.1). The analogous values for the four denatured samples were 4.60 Å. (4.60) and 10.35 Å. (9.95 to 10.6). The grouping of our tuberculin protein x-ray diffraction patterns according to their different degrees of denaturation is in good agreement with the physicochemical behavior of these preparations. For example, the tuberculin proteins which show an x-ray diffraction pattern characteristic for denaturation are lowest in solubility and antigenicity. Preparations of high solubility and antigenicity show diffuse x-ray diffraction rings such as are generally observed in undenatured proteins. Conversely it seems possible to use x-ray diffraction analysis for the elucidation of certain physicochemical changes in tuberculin proteins. Attempts at correlation of x-ray diffraction patterns with biological potency will be postponed until purer samples are available.

An attempt was made to analyze further the x-ray diffraction pattern by studying the width of the diffraction lines. It has been shown that the width of these lines is dependent upon the size of the particles under investigation (5). Since it is probable that the concomitant substances, when present in the above concentrations, have very little effect upon the particle size, such studies seemed likely to reveal reliable information, although the method had been originally used only for substances of strong x-ray absorption power and definite crystalline character. As had been mentioned before, three different tuberculin proteins (SOTT-b3, PPD-61, and PPD-49609) are available for which the

molecular weight has been determined according to the ultra-centrifugal method of Svedberg (13). The molecular weights vary between 13,500 and 17,000. The method, especially for such small sized particles, is not sufficiently exact to make sure that the three tuberculin proteins under investigation are of different molecular weight. Nevertheless, according to the different degrees of sharpness and intensity in the diffraction patterns, Preparations PPD-61 and PPD-49609 seem to be more hydrated and less x-ray-diffracting than SOTT-b3, which according to Katz (11) should influence the results.

For the determination of the particle size D, the equation

$$B = K \frac{\Lambda}{D \cos \Theta/2} \tag{1}$$

was used. The constant K is related to the shape of the cell unit. It is not likely that the cell unit is cubical in the case of SOTT-b3, since Seibert has found for this tuberculin protein a molar frictional ratio of 1.6. The validity of this assumption is further verified by a computation in which the ratios of $\sin^2 \Theta/2$ are found not to be of whole numbers. It is therefore proper to use the value 0.92 for the constant K (14). (In this equation B is the width of the diffraction line (or ring) at half maximum intensity, measured in radians from the specimen, $K = 0.92$, Λ is the wave-length of the monochromatic x-ray beam, and Θ is the diffraction angle as defined by Bragg's law.)

B, the width of the diffraction line at half maximum intensity, was graphically determined in all instances from photoelectric density graphs. Since the computations for the different tuberculin protein specimens are analogous, only the computation for one sample (SOTT-b3) will be recorded here. Fig. 4 shows the determination of the half maximum intensity for both diffraction rings from the photoelectric density graphs.

Computation—In our camera the film is flat, so that the diffracted x-ray strikes the film at an angle. It is necessary therefore to take this into account for an accurate determination of the width of the diffraction line. This may readily be done as follows (Fig. 5):

In Fig. 5, the incident x-ray beam striking the specimen at C is diffracted toward AEG. Point E is the point of maximum

intensity and A and G are points of half this intensity. AG is the half maximum intensity width and angle ACG (measured in radians) is equal to B in the above equation. $B = \delta - \gamma$; tan $\gamma = FA/CF$; tan $\delta = FG/CF$. FA and FG are obtained from the density curve of the diffraction ring (Fig. 4). CF is the specimen film distance in the camera, 37.7 mm. (We have shown by special experiments that when the thickness of the specimen was doubled, from 1.32 to 2.64 mm., by packing in superimposed holes of two microscopic slides instead of one slide, the width of the diffraction rings was not increased appreciably; the values of the half intensity
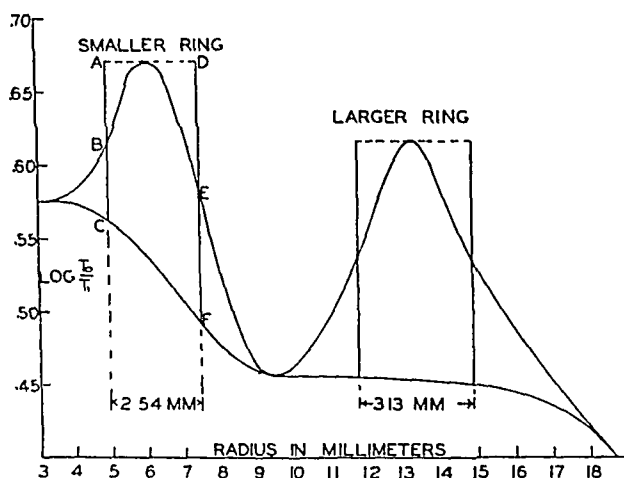
Fig. 4. Photometric density graph of SOTT-b3. Graphical determination of half brightness width. $AB = BC$; $DE = EF$; AD is the half brightness width. Log t_0/t_1 indicates density; mm. indicates distance from the center of the x-ray diffraction pattern.

width were 4.3 and 4.275 mm. respectively.) For the larger ring of tuberculin protein SOTT-b3 it was found that $FA = 11.74$ mm. and $FG = 14.86$ mm. Then tan $\gamma = 11.74/37.7 = 0.3114$ and $\gamma = 17° 18'$; tan $\delta = 14.86/37.7 = 0.3942$ and $\delta = 21° 31'$. $B = 21° 31' - 17° 18' = 4° 13' = 0.07359$ radians; $\Lambda = 1.54 \times 10^{-8}$ cm.; tan $2\Theta = FE/CF = 13.2/37.7 = 0.3502$, $2\Theta = 19° 18'$, and $\Theta = 9° 39'$. Substituting these values in Equation 1 and solving for D, we obtain

$$0.07359 = 0.92 \frac{1.54 \times 10^{-8}}{D \cos 9° 39'}; \quad D = 19.53 \text{ Å}.$$

Considering the smaller ring of the diffraction pattern, we find $\tan \delta = 7.48/37.7 = 0.1985$, $\delta = 11° 14'$; $\tan \gamma = 4.94/37.7 = 0.1310$, $\gamma = 7° 18'$. $B = 11° 14' - 7° 18' = 3° 56' = 0.06868$ radians; $\tan 2\theta = 6.10/37.7 = 0.1618$, $2\theta = 9° 12'$, and $\theta = 4° 36'$. Then,

$$0.06868 = 0.92 \frac{1.54 \times 10^{-8}}{D \cos 4° 36'}; \ D = 20.67 \text{ Å}.$$

Our results show that the diameters computed from the two diffraction rings are practically identical. This should indicate that the
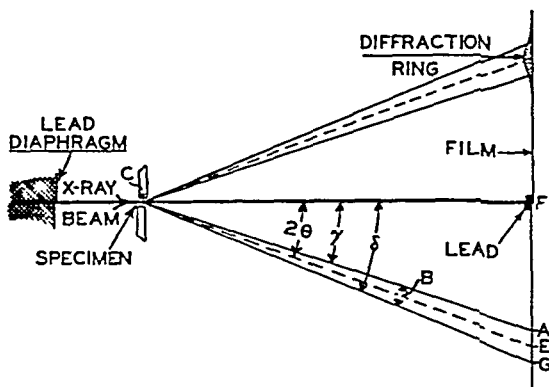
FIG. 5. Diagram showing how to compute the exact width of diffraction lines in radians from measurements made with a flat camera. C is the specimen, F the center of the film protected by lead, AEG is the diffracted x-ray beam, E the point of maximum intensity, A and G are points of half this intensity, AG is the half maximum intensity width, and angle ACG (in radians) $= B = K(\lambda/(D \cos \theta/2))$.

tuberculin protein particle has two almost equal dimensions in the two principal crystallographic directions. Clark and Rhodes (15) have drawn the same conclusions from similar results in rubber black carbon.

For tuberculin proteins PPD-61 and PPD-49609, diameters of 15.63 and 15.26 Å. were computed. These results agree with the molecular weights of these tuberculin proteins in that they fall into the same sequence as to magnitude. Nevertheless there is a possibility that the differences in the computed particle sizes are only the consequence of the influence of denaturation upon the

x-ray diffraction patterns of proteins.   According to Astbury and Lomax (9) and our own findings (12) denaturation causes a sharpening of the originally diffuse diffraction lines, which improves the exactness of the photometric readings and necessitates a decrease in the half intensity width.   Our formula shows that this function is inverse to the size of the particle.   Therefore it seems obvious that substances with diffuse rings—if the Scherrer method can be used at all for them—would give smaller particle sizes than the identical substances after sharpening of their lines following denaturation.   The differences in the particle sizes which we have found between the genuine and the denatured tuberculin protein are much too small to be explained by an appreciable degree of polymerization.   Furthermore we have been able to show the reversibility of the x-ray diffraction changes after reversal of heat denaturation (12).   It seems therefore probable that the discrepancy in the particle sizes is only partly real and that the higher values are more accurately determined.

A particle size of approximately 15 to 20 Å. diameter accounts for the colloidal character of the solutions of tuberculin protein. Its size comes rather close to the values which Scherrer (5) has found in Zsigmondy's amicroscopic colloidal gold.   Since the molecular weight of SOTT-b3 is about 17,000, it is understandable that the particles should be much smaller than those of other proteins, the molecular weights of which are multiples of 17,600 (16).

We have tried to find out whether the diameter of the particle is identical with the diameter of a tuberculin protein molecule. We followed in this instance the example of Zsigmondy (17), who used the formula of Reinganum (18) for the determination of the diameter of hemoglobin.   According to this formula $\sigma = 0.882 \times 10^{-8} \sqrt[3]{M/S_s}$ cm. (σ = diameter, M = molecular weight, S_s = specific weight at boiling point).   For M we can substitute 17,000, 15,700, and 14,500 respectively.   Since numerically the density is identical with the specific gravity, we can use $D = 1.33$ as determined by Seibert and coworkers (3).   According to Bull (19) heat denaturation, to be expected at the boiling point, changes the density of protein but little.

For the three tuberculin proteins SOTT-b3, PPD-61, and PPD-49609, diameters of 20.62, 20.08, and 19.56 Å. can be com-

puted in this way. The first of these values, 20.62 Å., is practically identical with the corresponding one found according to the Scherrer method, but all of our diameters, computed with the formula of Reinganum, vary within a narrow range. This seems to give additional support to our assumption that the Scherrer method gives best results in proteins when their x-ray diffraction lines are sharpened by denaturation.

These findings show, therefore, that it is possible to use the Scherrer method for the determination of the size of protein molecules and incidentally to use these results for the determination of the molecular weight of a protein of known density.

This work is being continued on different lines.

SUMMARY

1. X-ray diffraction patterns were taken of thirteen samples of analyzed tuberculin proteins.

2. After the effects of buffer salts, polysaccharides, and nucleic acids present as impurities are accounted for, the x-ray diffraction patterns of tuberculin proteins show spacings at 4.43 to 4.60 Å. and 9.64 to 10.6 Å. These spacings are within the range observed for other proteins under similar conditions.

3. In three samples of tuberculin proteins a determination of the particle size was made according to the method of Scherrer. Diameters of approximately 20 Å. were computed. These values are in good agreement with molecular diameters computed from the molecular weights and densities of the tuberculin proteins. This seems to be a partial justification of the assumption of crystallinity of tuberculin proteins which is the basis of our computation.

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CALCIUM IN THE ALIMENTARY TRACT OF THE RAT

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The belief that calcium is excreted through the intestine as part of a mechanism regulating the metabolic level for this element is founded largely on indirect evidence and has been recently challenged. Evidence indicating that calcium excretion into the gastrointestinal tract is negligible has been reported by Nicolaysen (11) working on dogs, by Christiansen (2) for the rabbit, by Johnson (7), Aub and coworkers (1), and McCance and Widdowson (10) for man, and by Henry and Kon (5) for the rat. The opposite view-point is upheld by Ross and Scriver (13) and Pugsley (12) who experimented with rats and by Cowell (4) who used rabbits.

We are reporting here a technique which we have used with rats for studying calcium absorption and also experiments in which this method has been applied to measure the amount of calcium found in the intestine under varying conditions. Instead of fasting the animals, as is often done in absorption experiments, we have kept the alimentary tract normally filled by feeding a diet which, while practically free of calcium, contained a liberal amount of roughage and was adequate in other respects.

EXPERIMENTAL

Rats weighing approximately 150 gm. which had been raised on the Yenching stock diet were fed *ad libitum* a low calcium diet (Table I) for a period of 4 days, the rats being kept in individual cages with false bottoms. 2 cc. of calcium lactate, containing 10.4 mg. of calcium, were then fed by stomach tube and the rats were killed after a definite time interval. The alimentary tracts were ligated, removed, and the contents of the several portions

of the tract as indicated were analyzed for calcium. In the control group, 2 cc. of distilled water were administered instead. The technique followed was in general that of Cori (3) as originally applied to the study of the absorption of sugars in the alimentary tract. The residues, removed from the alimentary tract by careful washing, were evaporated, ashed in silica dishes, and analyzed for calcium by the micromethod of Kramer and Tisdall (8).

In the control determinations, shown in Table II, when no calcium was fed, the amount of calcium found in the alimentary tract averaged 0.87 mg. per 100 gm. of body weight, which represented presumably the small amount of calcium contained in the diges-

<div align="center">

TABLE I

Low Calcium Diet

</div>

	per cent
Starch...	45
Cellulose*..	10
Lactalbumin†.....................................	15
Lard...	23
Salt mixture‡....................................	2
Cod liver oil....................................	5
Ca content......................................	0.017
P "	0.303

* Regenerated cellulose (Sylphrap Corporation).
† Lactalbumin 7HAA (The Dry Milk Company).
‡ Ca-free salt mixture (Adolph, W. H., Wang, C.-H., and Smith, A. H., *J. Nutrition*, **16**, 291 (1938)).

tive juices plus unabsorbed calcium from the diet. This figure was regarded as a blank and was subtracted in the case of the experimental animals from the calcium found in the intestinal tract per 100 gm. of body weight in calculation of the amount absorbed. The absorption coefficient is calculated in terms of mg. of calcium absorbed per hour per 100 gm. of rat body weight. Table II also shows the manner in which the unabsorbed calcium accumulates in different portions of the alimentary tract during the absorption process after definite intervals of time.

To determine the magnitude of the calcium "excretion" in the intestine, the method of analysis of alimentary contents was next applied to rats in which approximately the same amount of

calcium was injected intravenously instead of being administered by stomach tube. The calcium in this case was injected as boro-gluconate solution, 0.6 cc. being injected into the femoral vein;

TABLE II

Absorption of Calcium Administered As Lactate by Stomach Tube to Rats on Low Calcium Basal Diet

The figures represent amounts of calcium found in the alimentary tract after stated intervals of time.

Sex	Ca fed	Absorption period after feeding	Weight of rat	Ca found in alimentary tract					Ca absorbed per 100 gm. body weight per hr.
				Stomach	Small intestine	Cecum	Large intestine	Total	
	mg.	hrs.	gm.	mg.	mg.	mg.	mg.	mg.	mg.
M.	0		161	0 30	0 49	0 36	0 30	1.44	
"	0		151	0 19	0 62	0 40	0.26	1.46	
"	0		144	0 14	0 24	0 24	0 30	0 92	
"	0		136	0 17	0.44	0 33	0.38	1 32	
Average .									0.87
M.	10.4	1	162	0 28	4 49	0 43	0 22	5.42	3.9
F.	10.4		127	0 66	5.43	0 72	0 22	7.03	3.5
M.	10.4		135	1 14	4.80	0.51	0 38	6.83	3.5
"	10.4		137	1 97	3.38	0 85	0.48	6.68	3.6
Average...									3.6
M.	10.4	2	103	0 74	2.17	1.62	0.10	4.63	3.2
F.	10.4		108	0 74	1.49	2.99	1.69	6.91	2.0
M.	10.4		121	0 87	5 05	0 70	0.62	7.24	1.7
"	10.4		138	0 17	1 86	3 58	1.23	6.84	1.7
Average....									2 2
M.	10.4	3	112	0 19	2 22	1 57	2.29	6.27	1.5
"	10.4		113	0 43	1 35	1 52	2 68	5.98	1.6
"	10.4		119	0 12	0 98	3 99	1.03	6.12	1.5
"	10.4		140	0.13	0 45	4.20	1 34	6.12	1.3
Average... .									1.5

the solution was prepared as recommended by Macpherson and Stewart (9). The rats were then killed after an interval of 3 hours. In one set of experiments the animal was lightly anes-

thetized and ligatures were placed on the intestine above and below the cecum immediately before the injection. This was for

TABLE III

Intestinal Calcium in Rats Fed Low Calcium Diet, Killed 3 Hours after Single Intravenous Injection of Calcium Borogluconate

Sex	Ca injected	Weight of rat	Ca found in intestinal tract				Total per 100 gm. body weight
			Small intestine	Cecum	Large intestine	Total	
	mg.	gm.	mg.	mg.	mg.	mg.	mg.
M.	0	202	0.54	1.07	0.45	2.06	1.02
"	0	225	1.43	2.12	2.63	6.18	2.75
"	0	253	1.00	2.54	2.58	6.12	2.42
F.	0	176	0.97	1.98	0.13	3.08	1.75
M.	0	183	0.88	1.60	0.94	3.42	1.87
F.	0	179	0.70	1.76	1.18	3.64	2.03
"	0	200	1.44	2.27	2.40	6.11	3.05
M.	0	218	0.58	0.98	0.46	2.02	0.93
Average....................................							1.98
M.	9.18	195	0.69	1.41	1.23	3.33	1.70
"	9.18	169	1.40	2.75		4.15	2.45
F.	9.18	150	0.92	2.50		3.42	2.28
"	9.18	164	0.48	1.82		2.30	1.40
"	9.18	206	1.02	2.32		3.34	1.62
"	9.18	194	0.80	2.25		3.05	1.57
M.	9.18	215	1.18	1.74	1.17	4.09	1.90
Average....................................							1.84
M.*	9.18	214	1.45	3.39	1.52	6.36	2.98
" *	9.18	198	1.35	0.85	0.32	2.50	1.26
" *	9.18	223	1.08	2.44	0.85	4.37	1.96
F.*	9.18	148	0.66	0.86	0.28	1.80	1.22
" *	9.18	195	1.59	1.10	0.77	3.56	1.83
M.*	9.18	253	1.38	0.80	0.38	2.56	1.01
" *	9.18	224	1.82	1.03	0.66	3.51	1.57
Average....................................							1.69

* Ligatures were placed above and below the cecum.

the purpose of preventing whatever calcium may be excreted from being transported from one portion of the intestinal tract

to the next and to aid in locating the portion of the intestinal tract where "excretion" took place. Results, as shown in Table III, indicate that under the conditions employed the experimental animals showed no appreciable increase in the amount of calcium found in the intestinal tract. Similar results, not recorded here, were obtained when the time interval after injection was reduced to 2 hours and also to 1 hour.

TABLE IV

Intestinal Calcium in Rats Fed Low Calcium Diet with Daily Addition of Calcium Subcutaneously, Killed 8 Hours after Single Intravenous Injection of Calcium Borogluconate

Sex	Ca injected	Weight of rat	Ca found in intestinal tract				
			Small intestine	Cecum	Large intestine	Total	Total per 100 gm. body weight
	mg.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
F.	14.3	173	1.09	1.33	0.57	2.99	1.73
"	14.3	205	0.75	2.60	3.20	6.55	3.20
"	14.3	159	0.96	1.65	2.72	5.33	3.35
M.	14.3	188	1.68	5.38	8.20	15.26	8.01*
"	14.3	176	0.60	1.06	1.42	3.08	1.74
"	14.3	206	1.18	1.38	1.19	3.75	1.82
"	14.3	181	0.76	1.32	0.76	2.84	1.57
"	28.6	164	2.38	1.45	0.80	4.63	2.82
"	28.6	171	1.68	1.62	0.84	4.14	2.42
"	28.6	134	1.57	0.39	0.95	2.91	2.16
Average...							2.31

* Not included in the average.

It may be noted, however, that the rats receiving calcium by injection had been fed for a brief period of time on a low calcium diet. This diet was so low in calcium that such an animal, unsaturated with respect to calcium, might so rapidly utilize all injected calcium to restore the depleted body calcium that no possible excess would be left which could be excreted. The same experiment was therefore repeated except that during the 4 day period, when the animal was on the low calcium diet, 9.2 mg. of calcium, not less than the rat's daily calcium requirement, were injected subcutaneously daily in the form of borogluconate. As

in the previous experiment, on the experimental day a single intravenous injection of calcium as borogluconate was made; 3 hours later the animal was killed. In this case a more concentrated solution was used so that intravenous injections of 14.3 and 28.6 mg. of calcium were possible. The results, shown in Table IV, when compared with the figures for the control group, again indicate no significant increase of intestinal calcium.

DISCUSSION

The data obtained indicate that under controlled conditions a constant amount of calcium is to be found in the intestinal residues and that the method of intestinal analysis may be applied to the measurement of calcium absorption. We found that 4 days were sufficient to remove from the intestinal tract the calcium from previous feedings and that it was not necessary to use the device of removing the cecum adopted by Innes and Nicolaysen (6). The cecum, as shown in Table II, does not store unusual amounts of calcium and thus interfere with the accuracy of the results.

The absorption coefficients calculated indicate a diminution of the absorption rate over longer periods of time. These figures, it should be noted, are obtained with small amounts of calcium. With large amounts of sugar and with more concentrated solutions, Cori (3) found the absorption coefficient constant for any one substance.

Our particular interest is in the amount of the so called intestinal excretion. Estimates in the literature have led to the belief that the amount of calcium contained in the normal secretions of the gastrointestinal tract is considerable. Our results, however, suggest that, at least in the case of the rat, it is rather low (Table II). In balance experiments with rats fed calcium-free diets similar to the diet here employed, we have found (unpublished data) the total fecal calcium per 100 gm. of rat body weight to average only 0.6 mg. per day.

In the final experiment the calcium injected into the rat was 14 and 28 mg. per day, whereas the daily calcium requirement of the 150 gm. rat may be reckoned as 8 to 10 mg. per day. This therefore represented a liberal supply of calcium and in spite of this there was no significant excretion of an excess into the intes-

tine. These experiments indicate that, while there is a small amount of calcium normally excreted into the gastrointestinal tract, this amount, however, is not affected by variations in the plasma calcium level.

SUMMARY

A microtechnique is reported for the measurement of calcium absorption rates in the rat.

When calcium as borogluconate is injected intravenously into rats fed a low calcium diet, there is no significant increase in the calcium content of the intestinal tract. It may be concluded that in the rat the intestinal tract does not excrete calcium in amounts which are related to the metabolic calcium level.

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STUDIES ON THE CHEMICAL CONSTITUTION OF THE ANTIGENIC SUBSTANCE IN ALCOHOLIC TISSUE EXTRACTS CONCERNED IN THE SERUM DIAGNOSIS OF SYPHILIS

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The first antigens used in the Wassermann test were aqueous extracts of syphilitic tissues and especially of the livers of congenitally syphilitic fetuses. It was soon discovered that alcoholic extracts of these tissues were also antigenic. This was followed by the discovery that aqueous and alcoholic extracts of guinea pig liver or other non-syphilitic tissues of human and animal origin likewise carried the antigenic principle. At present antigens for the complement fixation and flocculation tests for syphilis are usually prepared of alcoholic extracts of beef heart. It is commonly thought that the antigenic principle consists of alcohol-soluble lipids, but numerous attempts to isolate and identify them have been not only largely unsuccessful but have led to widely divergent results and opinions. That the isolation and identification of the antigenic principle in tissue extracts is highly desirable is quite evident, since it might result in the preparation of antigens of more uniform potency, especially if a synthetic preparation were available. The present investigation is concerned with a method of study not hitherto employed to the best of our knowledge.

Review of Literature

No attempt will be made to review the voluminous literature on the subject but brief mention must be made of some of the

reported work to indicate the divergent opinions on the subject. Thus Neymann and Gager (1) found by fractionating beef heart muscle with lipid solvents that both the lecithin and the diamino-phosphatide obtained were strongly antigenic while the cephalin was without antigenic activity. But 15 years later Kiss (2) prepared a cephalin fraction which was found highly antigenic.

Lecithin was regarded as the actively antigenic substance by Browning, Cruickshank, and McKenzie (3) because of its solubility characteristics. Scaltritti (4) also thought that the active antigenic principle was a lecithin because it was precipitated by cadmium chloride, and Eagle (5) found the same percentage of nitrogen in the active lipids of beef heart adsorbed on sterols as is present in lecithin. But Weil and Ritzenthaler (6) were unable to find the active antigenic substance in cadmium chloride precipitates. MacLean and MacLean (7) referring to unpublished work, state that they and Dudgeon were able to prepare very active solutions that were practically lecithin-free and Fischer (8) concludes that not only is the active principle not a lipid but with Günsberger (9) has been able to isolate from various tissues antigenic fractions that were nearly insoluble in chloroform and alcohol.

The difficulty of isolating chemically pure phosphatides has been indicated by Levene and Komatsu (10) and the ease with which extraneous substances may be carried along by precipitated phosphatide has long been known. As emphasized by Christensen (11), Folch and Van Slyke (12), and others, such factors have undoubtedly entered into the results referred to above.

Scope of Investigation

The methods of investigation hitherto employed have been to secure and identify chemically as accurately as possible various lipoidal substances extractable from tissues (especially beef heart) by alcohol, ether, acetone, and other solvents, determining the antigenic activity of each by complement fixation and flocculation tests with syphilitic serum, with the hope of ascertaining the chemical nature of the substance which is antigenic in these serological reactions.

In our investigation we have utilized the precipitates forming

in the mixture of Kahn standard antigen with syphilitic serum in optimum proportions. These precipitates were used on the assumption that syphilis antibody or reagin will precipitate the active antigenic substance in a relatively pure state and with the minimum of loss. Such precipitates are far from pure chemical entities, but the results of preliminary studies have indicated that they contain practically all of the active antigenic substances present in the Kahn standard antigen.

<div align="center">EXPERIMENTAL</div>

Kahn standard antigen was employed not only because it produces marked precipitation with syphilitic serum, but likewise because it is also antigenic in the Kolmer complement fixation test which was employed for measuring the antigenic activity of the substances recovered from the precipitates. Furthermore, all of the antigens of beef heart commonly employed in complement fixation and flocculation tests in syphilis, being essentially alcoholic extracts reinforced with cholesterol, are practically identical in the principles of preparation.

Preliminary Kahn tests with each lot of serum were conducted to determine the amount of antigen required for securing maximum precipitation and the serum, in lots of about 150 cc., was then treated with the optimum amount of antigen. After standing in the refrigerator overnight, the precipitate, secured by centrifuging, was washed twice with 0.8 per cent sodium chloride to remove adherent serum, dried *in vacuo* over calcium chloride, and weighed.

The dried precipitate was extracted with four 20 cc. portions of a mixture of 3 parts of absolute alcohol and 1 part of absolute ether, and the residue, after being washed with water until salt-free, was dried *in vacuo* and weighed. This residue was non-antigenic and gave a positive biuret reaction. Presumably it was composed of the antibody globulin of the syphilitic serum but no further work was done with it.

The combined alcohol-ether extracts were concentrated to about 3 cc. *in vacuo* and treated with 25 cc. of redistilled acetone. The mixture was allowed to stand in the refrigerator overnight, the acetone was decanted, and the residue, after being kneaded with acetone, was dissolved in fresh alcohol-ether mixture and

again precipitated with acetone. The combined acetone solutions (Fraction A) were measured and aliquots taken for determinations of cholesterol content and antigenic activity.

The acetone-insoluble portion (Fraction B) was further subdivided, in the customary manner, into an alcohol-soluble, ether-insoluble part (Fraction C), an ether-soluble, alcohol-insoluble part (Fraction D), and an alcohol-, ether-soluble part (Fraction E).

Cholesterol was determined by the method of Man and Peters (13), nitrogen by micro-Kjeldahl, phosphorus by the method of Fiske and Subbarow (14), and choline by the method of Beattie (15) after barium hydroxide hydrolysis according to Johnston et al. (16). Qualitative tests for diaminophosphatides were made by the Thannhauser and Setz method (17). Antigenic and anticomplementary activities were determined according to the Kolmer complement fixation test (18) in which an anti-sheep hemolytic system was employed with a primary incubation of 15 to 18 hours at 6–8° followed by a water bath at 37° for 10 minutes.

Results

Five different lots of serum,[1] comprising in all about 4 liters were worked up separately; all lots gave similar results and Tables I to III, representing the data obtained from the precipitation of 560 cc. of serum with 77.6 cc. of Kahn standard antigen are representative of the group.

In Table I are listed the yields of the various fractions obtained from the precipitate, including that of the antibody globulin recovered. The amount of the latter falls within the range given in the literature (19) and it is interesting to note, as a measure of the sensitivity of the Kahn flocculation reaction, that it caused a precipitate of about 20 times its own weight. The acetone extract (Fraction A) was but feebly antigenic (Table II) and its cholesterol content (453 mg.) accounted for nearly all of the cholesterol present in the original antigen (465 mg.).

Tables II and III show that an alcoholic solution of Fraction B containing 5 mg. per cc. was as potent an antigen as the original

[1] We are indebted to Miss Carola E. Richter of the Pennsylvania Department of Health Laboratories for our supplies of strongly positive syphilitic serum.

Kahn antigen which contained 14.8 mg. per cc. of acetone-insoluble material, indicating that a 3-fold concentration of the active principle was obtained by the method here employed.

TABLE I

Amount of Precipitate and Its Various Fractions from 560 Cc. of Strongly Positive Syphilitic Serum Treated with 77.6 Cc. of Kahn Standard Antigen

Precipitate and fractions	Total weight	Weight per cc. antigen
	gm.	*gm.*
Original ppt.	1.010	
Protein content	0.050	0.0009*
Cholesterol (Fraction A)	0.453	
Total phospholipid (Fraction B)	0.387	0.005
Ether-insoluble substance (Fraction C)	0.034	
Absolute alcohol-insoluble substance (Fraction D)	0.055	
Alcohol-soluble substance (Fraction E)	0.281	
Acetone-insoluble lipids in Kahn antigen		0.0148

* Per cc. of serum.

TABLE II

Results of Antigenic Titrations by Kolmer Complement Fixation Technique

Antigens	0.5 cc. of dilutions										
	1:100	1:200	1:400	1:800	1:1200	1:1600	1:2400	1:3200	1:4800	1:6400	1:9000
Kahn standard	4*	4	4	4	4	4	4	1	1	–	–
Fraction A	2	1	1	–	–	–	–	–	–	–	–
" B†	4	4	4	4	4	4	3	2	–	–	–
" C†	–	–	–	–	–	–	–	–	–	–	–
" D†	4	4	4	4	4	4	4	4	4	4	3
" E†	4	4	4	4	4	4	4	3	3	–	–

* 4 = ++++, 3 = +++, 2 = ++, 1 = +.
† Made up to contain 0.005 gm. per cc. with the addition of sufficient cholesterol to give a 0.6 per cent solution.

Of the three components of Fraction B, each being made up to contain 5 mg. per cc., the ether-insoluble portion, Fraction C, was without antigenic activity while both Fractions D and E were about equally potent and compared favorably with the original antigen (Tables II, III). Nitrogen values of 2.9 to 3.1

per cent and phosphorus values of 3.1 to 3.4 per cent were obtained for Fraction C. It was strongly positive for diaminophosphatide (17), while Fractions D and E were completely negative. Apparently diaminophosphatides are not antigenic.

Fractions D and E gave similar phosphorus and nitrogen values varying from 3.8 to 4.1 per cent for phosphorus and from 0.9 to 1.1 per cent for nitrogen. Fraction D was negative for choline, while Fraction E contained 7.5 to 8.0 per cent of choline, an amount which accounted for all of the nitrogen found in Fraction E. The iodine number (Hanus) of Fraction E was 81; the al-

TABLE III

Results of Anticomplementary Titrations by Kolmer Complement Fixation Technique

Antigens	0 5 cc. of dilutions					
	1:5	1.10	1 20	1:30	1·40	1·60
Kahn standard.... .	—*	—	—	—	—	—
Fraction A........	—	—	—	—	—	—
"　　B†.... .	—	—	—	—	—	—
"　　C†........　　.	—	—	—	—	—	—
"　　D†	4	4	4	4	4	2
"　　E†	—	1	2	2	1	—

* — = no inhibition of hemolysis, 1 = slight inhibition, 2 = marked inhibition, 4 = complete inhibition.

† Made up to contain 0.005 gm. per cc. with the addition of sufficient cholesterol to give a 0.6 per cent solution.

cohol-insoluble portion, Fraction D, has thus far not been obtained in sufficient quantity for other data.

Acid hydrolysis of Fraction E was incomplete, an unexpected finding since lecithin is readily hydrolyzed by acid. Anderson (20) experienced similar difficulty with carbohydrate-containing lipids; however, our incomplete acid hydrolysates were negative for reducing sugars.

For alkaline saponification 30 cc. of an alcoholic solution of 150 mg. of Fraction E and 300 mg. of potassium hydroxide were refluxed for 4 hours. The mixture was concentrated to about 10 cc., diluted with 3 volumes of water, and extracted with three portions of ether. The combined ether extracts, washed free

of alkali, were evaporated to dryness; the residue was dried *in vacuo* and weighed as unsaponifiable.

The aqueous alkaline solution together with the wash water was acidified and extracted three times with ether. After the extract was washed free of acid, the ether was evaporated *in vacuo*. The mixed fatty acids were preserved in the dark *in vacuo*.

The aqueous acid solution and wash water were concentrated to a small volume and extracted with three 10 cc. portions of absolute alcohol. To the combined extracts was added a saturated barium hydroxide solution to alkalinity (phenolphthalein), and after the solution had stood in the cold overnight, the grayish amorphous precipitate was removed by centrifuging. It was reprecipitated twice from water by adding 2 volumes of absolute alcohol. The final product was a white amorphous powder completely soluble in water. It was dried and weighed as barium glycerophosphate.

$C_3H_7O_6PBa \cdot 2H_2O$. Calculated. Ba 39.94, P 9.03
                       Found.   " 39.78, " 8.78

A typical alkaline hydrolysis gave the following results: unsaponifiable 3.3 per cent; glycerophosphoric acid 17.4 per cent; mixed fatty acids 68.0 per cent, iodine number 111; choline (from $Ba(OH)_2$ hydrolysis) 7.6 per cent.

DISCUSSION

The high antigenic activity of Fractions D and E raises a point of interest. The method employed in their isolation from Fraction B was essentially the classical one for the isolation of cephalin and lecithin. Since both fractions are equally potent per unit weight and both are as antigenic as the original Kahn antigen, it is obvious that neither cephalin nor lecithin is directly responsible for the antigenic activity. The conclusion seems justified that both fractions carry, by adsorption or otherwise, the active antigenic principle which is not identical with either. The close similarity of the nitrogen and phosphorus results in both fractions and the fact that the nitrogen values are only about 60 per cent of theory and that the choline figures account for all of the nitrogen in the lecithin fraction support the assumption that both fractions are mixtures of a known phosphatide which is

antigenically inert and an unknown non-nitrogenous, phosphorus-containing substance.

The phosphorus, fatty acid, and glycerophosphoric acid values are greatly in excess of the requirements for the presence of a 60 per cent lecithin content of Fraction E and the excess of each allows for the existence of an ester of glycerophosphoric acid containing 2 molecules of fatty acid. As material becomes available, attempts will be made to determine the nature of this contaminant which appears in both the cephalin and lecithin fractions as well as its relationship to antigenic activity.

The iodine number of the mixed fatty acids indicates the presence of an acid of a greater degree of unsaturation than oleic. Because of lack of material the acids were not characterized; however, the relationship of the degree of unsaturation of fatty acids to antigenic activity is uncertain. In none of the procedures for the preparation of tissue extracts for antigenic purposes is any effort made to prevent oxidation. Indeed, in some of the methods it is directed that excess of precipitant be removed from the acetone-insoluble lipids by fanning, and yet the subsequent alcoholic extracts are highly antigenic.

SUMMARY

1. By an analysis of the precipitate formed in the Kahn flocculation reaction an attempt has been made to determine the active antigenic principle in alcoholic beef heart extracts.

2. The precipitate consists of cholesterol, reagin protein, and mixed phosphatides. The latter were separated into an ether-insoluble, alcohol-soluble diaminophosphatide which is inactive and two other fractions both of which are equally potent per unit weight and compare well with the original Kahn antigen.

3. Although the two antigenically active fractions have the solubility characteristics of a cephalin and a lecithin respectively, yet the analytical values indicate that they are mixtures, each containing about 40 per cent of a non-nitrogenous, phosphorus-containing substance. The available evidence indicates that the antigenic activity cannot be a property of either lecithin or cephalin but is due to an unknown substance adsorbed in equal proportions by both.

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COMPARATIVE STUDIES OF THE METABOLISM
OF THE AMINO ACIDS

IX. GLYCINE PRECURSORS. AVAILABILITY OF N-ETHYLGLYCINE AND GLYCOLIC ACID FOR THE SYNTHESIS OF HIPPURIC ACID BY THE RABBIT

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In a previous study of the availability of the N-methyl derivatives of glycine for the synthesis of hippuric acid it was found that the monomethyl derivative (sarcosine) greatly increased the rate of excretion of hippuric acid when administered with benzoic acid, whereas the more completely methylated derivatives, N,N-dimethylglycine and betaine, did not (1). The utilization of sarcosine in the detoxication mechanism was interpreted as evidence of the biological conversion of sarcosine to glycine. Further evidence of the biological transformation of sarcosine to glycine has been obtained recently in experiments of widely different nature. Bloch and Schoenheimer (2) found that isotopic glycine was deposited in the tissue proteins of rats fed N^{15} sarcosine, and Borsook and Dubnoff (3) found that sarcosine could furnish glycine for the synthesis of glycocyamine by rat kidney slices. N-Methylglycine can thus be utilized in lieu of glycine, just as the N-monomethyl derivatives of certain of the essential amino acids can replace the corresponding unsubstituted amino acid for purposes of growth.

It thus becomes of interest to determine what effect blocking of the amino group with an alkyl group somewhat larger than methyl would have on the utilization of the amino acid. With one exception, growth studies with N-alkyl essential amino acids have been confined to the N-methyl derivatives (4, 5). Carter and Handler (6) have reported that the N-*ethyl* derivative of *dl*-phenylalanine could not replace the unsubstituted amino acid for

growth purposes, whereas the N-methyl derivative of *l*-phenylalanine could. This would suggest that N-alkyl groups other than methyl are not readily removed from the α-amino group of the amino acid.

The present experiments were undertaken to determine whether N-ethylglycine could yield glycine in amounts which would be available for the synthesis of hippuric acid, as does sarcosine (1). It was found that the oral administration of N-ethylglycine, in contrast to that of sarcosine, resulted in no marked increase in the rate of excretion of hippuric acid by rabbits given benzoate.

Experiments were also made with glycolic acid (the hydroxy analogue of glycine) to see whether this compound might be a precursor of glycine and would thus be available for hippuric acid synthesis. The α-hydroxy analogues of many of the essential amino acids can replace the corresponding amino acids in a deficient diet and can support the growth of rats, thus indicating the conversion of the α-hydroxy analogue to the amino acid (4). Griffith and Lewis (7) included glycolic acid in the series of possible glycine precursors which they studied. Two experiments with this compound gave negative results; no increase in the rate of excretion of *total benzoic acid* was obtained when glycolic acid was given with benzoate to rabbits. Later, Griffith (8, 9), in preliminary reports, stated that the toxicity of benzoate fed to young rats was markedly decreased by glycolic acid, and (8) that "the addition of sodium glycollate to the toxic benzoate diet afforded practically the same protection as that furnished by glycine itself." Although this statement suggests a possible conversion of glycolic acid to glycine, and has been considered as evidence of this (4), it must be remembered that the resumption of growth of benzoate-poisoned rats when fed glycolic acid, although suggestive, is not conclusive evidence of the conversion of glycolic acid to glycine, since an increase in glycine production is not shown directly. The possibility that the decrease in toxicity of benzoic acid might have been due to factors other than the conversion of glycolic acid to glycine is not excluded; *e.g.*, it is conceivable that glycolic acid might be a precursor of glucuronic acid and might thus make available more of this detoxification agent for the conjugation of benzoic acid in the rat. No determinations of the benzoic acid derivatives in the urine of the rats were reported (8, 9).

It was thus of interest to study again the effect of glycolic acid on the rate of excretion of *hippuric acid* in the rabbit, hippuric acid being determined by the method of Griffith (10) which was developed after the earlier experiments of Griffith and Lewis (7).

<div align="center">EXPERIMENTAL</div>

N-Ethylglycine was prepared from glycine by the methods of Cocker (11) and of Cocker and Lapworth (12). Nitrogen analyses were close to theoretical, attesting the purity of the compound. The purity of the glycolic acid (Eastman) was checked by titration. The N-ethylglycine was administered as such and the glycolic acid was neutralized before feeding, as described subsequently.

The experimental procedure and methods of analysis used in the previous study of this series (1) were employed, and the experiments were carried out with the same animals.

The *rate of excretion* is shown by the percentage of the administered benzoic acid which is excreted in the first 6 hours. Values for the succeeding 18 hour periods indicate only completeness of the recovery of the administered benzoic acid and are omitted from Tables I and II, since we are concerned primarily with *rate* of excretion as an index of availability of glycine. No correction has been made for the small, constant amount of benzoic acid which is normally eliminated in a 6 hour period by these rabbits. This normal excretion was relatively constant, amounting to less than 100 mg. per day, and in the 6 hour period to only 18 mg. The difference resulting from applying this correction to all the figures of Tables I and II would be negligible. Creatinine determinations were made to check completeness of urine collections. These values, as well as those for total nitrogen, are also omitted, as they do not bear directly on this study.

<div align="center">DISCUSSION</div>

Data from experiments with N-ethylglycine are presented in Table I. From the figures showing the percentage of administered benzoic acid excreted as hippuric acid in the 6 hour period, it can be seen that the administration of N-ethylglycine with benzoate resulted in no significant increase in the rate of hippuric acid excretion, in contrast to the marked effect of sarcosine or

glycine. Thus when an ethyl group was substituted for the methyl group of sarcosine, either the conversion of the compound to glycine did not occur or occurred so slowly that the glycine formed could not be detected by the method used in this study.

TABLE I

Excretion of Benzoic Acid, Total and As Hippuric Acid, in 6 Hour Period Immediately Following Ingestion of Sodium Benzoate Alone and with Glycine, Sarcosine, or N-Ethylglycine

The benzoic acid, 0.66 gm. per kilo of body weight, was administered orally as the sodium salt.

Rabbit No.	Benzoic acid excreted as				Compound fed	Benzoic acid given	
	Hippuric acid		Total				
	gm.	per cent intake	gm.	per cent intake		moles	gm.
1	1.114	62.5	1.284	72.0	Sarcosine	3	1.782*
	0.500	21.3	0.763	32.5		0	2.343
	0.723	30.8	0.971	41.4	N-Ethylglycine	3	2.343
	0.573	24.4	0.893	38.0		0	2.343
	1.841	78.5	1.888	80.4	Glycine	3	2.343
3	1.300	85.6	1.348	88.8	"	3	1.518*
	1.258†	74.6	1.325	78.6	Sarcosine	3	1.683
	1.116†	66.2	1.225	72.7	"	3	1.683
	0.766†	45.5	0.912	54.1		0	1.683
	0.845†	50.2	1.041	61.8	N-Ethylglycine	3	1.683
	0.742	44.1	0.887	52.7		0	1.683
4	0.889	57.2	1.073	69.1		0	1.551
	1.340	86.3	1.377	88.7	Glycine	3	1.551
	0.863	55.6	1.031	66.5		0	1.551
	0.952	61.3	1.176	75.7	N-Ethylglycine	3	1.551
	1.246	80.2	1.307	84.1	Glycine	3	1.551

* With the exception of this experiment, which is included for comparison, the values represent consecutive experiments, at least 5 days apart. Since the animals increased in weight, the absolute amount of benzoic acid fed was increased in the later experiments.

† These values were checked by the determination of amino nitrogen after hydrolysis of the ether-extractable material, as described in the earlier paper (1).

It thus appears that although the rabbit can convert the monomethylated glycine compound to glycine and can use it for detoxification of benzoic acid, it cannot readily convert and use glycine which has had an ethyl group substituted for one of the

hydrogens of the amino group.   Bloch and Schoenheimer (2) have shown that sarcosine is transformed to glycine by direct de-methylation; *i.e.*, that, during the biological conversion of sar-cosine to glycine, the nitrogen originally attached to the carbon chain is not replaced.   The transformation of sarcosine to glycine is a rapid one (1, 2).   That conversion of N-ethylglycine to glycine is not indicated by our experiments, and that conversion of N-ethylphenylalanine to phenylalanine does not take place (6) would suggest that N-alkyl groups other than methyl are not readily removed from the amino acid molecule.   Perhaps the presence of the larger group has made it impossible for some enzyme system to react with the substrate, or has so interfered with the reaction that the amount "deethylated" cannot be detected by the ex-perimental procedures.

The results of experiments with glycolic acid are presented in Table II.   The data are from consecutive experiments, at least 5 days apart.   It will be noted, that although results with Rabbit 1 were indecisive, those with Rabbits 2 and 4 are highly sugges-tive.   In one experiment with Rabbit 2, the administration of glycolic acid was followed by an increased hippuric acid excretion corresponding to 53.6 per cent of the ingested benzoate, whereas, when this rabbit was given benzoate alone, the hippuric acid excreted was only 39 per cent of the benzoate intake in the 6 hour period following administration.   In repeated experiments,[1] this rabbit has failed to excrete more than about 40 per cent of the ingested benzoate as hippuric acid in the 6 hour interval when no glycine was given.   Although the increases in the rate of excretion found are much less than those noted with sarcosine, they are beyond the range of variations found when benzoate was given alone.   It is apparent, therefore, that glycolic acid cannot be converted to glycine to as great an extent, or as rapidly, as sar-cosine.   It was thought that perhaps administration of larger amounts would more readily demonstrate the effectiveness of glycolic acid as a glycine precursor in this type of experiment. In one experiment with Rabbit 4, 6 moles of glycolic acid were given per mole of benzoic acid.   The experiment, however, was unsatisfactory, as a severe diarrhea resulted.   However, the rate

[1] More extensive control values with this animal are given in the pre-vious paper (1).

of excretion of hippuric acid was increased from 41 to 51 per cent of the intake. Another experiment with Rabbit 4 gave an increase in the rate of excretion of hippuric acid from 40 to 54 per

TABLE II

Excretion of Benzoic Acid, Total and As Hippuric Acid, in 6 Hour Period Immediately Following Ingestion of Sodium Benzoate Alone and with Glycine or Glycolic Acid

The benzoic acid, 0.66 gm. per kilo of body weight, was administered orally as the sodium salt. Unless otherwise indicated, the glycolic acid was fed as the sodium salt.

Rabbit No.	Benzoic acid excreted as				Compound fed		Benzoic acid given
	Hippuric acid		Total				
	gm.	per cent intake	gm.	per cent intake		moles	gm.
1	1.522	78.2	1.589	81.6	Glycine	3	1.917*
	0.556	27.2	0.869	42.5	Glycolic acid	3	2.046
	0.588	28.3	0.945	45.5		0	2.079
	0.705	34.0	0.985	47.4	Glycolic acid	3	2.079
2	1.588	81.5	1.658	85.1	Glycine	3	1.917
	1.061	53.6	1.328	67.0	Glycolic acid	3	1.980
	0.793	39.4	1.069	53.2		0	2.013
	0.944	46.9	1.288	64.0	Glycolic acid	3	2.013
4	1.246	80.2	1.307	84.1	Glycine	3	1.551
	0.720	41.2	0.913	52.2		0	1.749
	0.895	51.2	1.405	80.4	Glycolic acid	6†	1.749
	0.747	40.4	1.010	54.7		0	1.848
	0.999	54.1	1.295	70.1	Glycolic acid	3	1.848
	0.700	35.4	0.860	43.4		0	1.980
	0.906	45.8	1.042	52.7	Glycolic acid‡	3	1.980
	0.989	50.0	1.071	54.1	" " ‡	3	1.980
	0.770	38.9	0.890	45.0	Glucose	1	1.980
	0.660	33.4	0.823	41.6		0	1.980

   * Since the animals increased in weight, the absolute amount of benzoic acid fed increased as the experiments progressed.

   † An extra 3 moles of glycolic acid was given the animal 3 hours before the feeding of the benzoate plus 3 moles of glycolic acid. Severe diarrhea resulted.

   ‡ One-third of the glycolic acid neutralized with ammonia, as described in the text.

cent of the intake after 3 moles of glycolic acid. In two experiments, 1 mole of ammonia was given with 3 moles of glycolic acid to see whether the effect of glycolic acid could be increased if the

ammonia required for amination were also supplied. One-third of the glycolic acid was neutralized with ammonium hydroxide, the remainder with sodium hydroxide. The rate of excretion obtained was no greater than when 3 moles of sodium glycolate were fed. However, increases in rate of excretion of hippuric acid were again observed, 10 per cent of the intake of benzoate in one case and 15 per cent in the other. Although these effects were not as great as could be demonstrated with sarcosine, we feel that they are significant.

Although the addition of ammonia to the glycolic acid did not increase the effect of glycolic acid on the rate of excretion of hippuric acid as measured by the excretion of hippuric acid in the 6 hour period following benzoate administration, it was interesting to note that the total recovery of hippuric acid in the 24 hour period following ingestion was much greater when ammonia was given with the glycolic acid, the values during this period approximating the values noted when glycine itself had been administered. Thus in the last four experiments with Rabbit 4 (Table II), benzoic acid excreted as hippuric acid in the 24 hour period following ingestion amounted to 93.6 and 96.4 per cent of the administered benzoate when ammonium glycolate was given, 85.3 per cent when glucose was fed with the benzoate, and 83.9 per cent when benzoate alone was given. These values may be compared with those obtained when glycine was given with benzoate (99.5 per cent) and when glycolic acid, without ammonia, was the experimental compound (76.4 per cent).

That the effect of glycolic acid was not due to an enrichment of the liver through possible formation of carbohydrate was shown by giving 1 mole of glucose (instead of 3 moles of glycolic acid) per mole of benzoate. No marked increase in the rate of excretion of hippuric acid was noted.

In experiments of this type, a positive result is of much greater significance than a negative one. Of a number of compounds that have been tested (1, 7) only glycine and sarcosine have been found to increase the rate of excretion of hippuric acid. The biological conversion of sarcosine to glycine has been confirmed (2, 3). For these reasons it is felt that the effect of glycolic acid in these experiments is probably due to its ability to serve as a source of glycine.

Milhorat and Toscani (13) have studied the effect of glycolic acid on the excretion of creatine in patients with progressive muscular dystrophy and found that the ingestion of glycolic acid was followed by a small increase in the output of creatine which was considerably less than (about one-third) that produced by equivalent amounts of glycine. Their results suggest the possibility that glycolic acid can be converted into glycine in the human subject.

The present experiments, in conjunction with those of Milhorat and Toscani (13), Griffith (8, 9), and unpublished results of White,[2] afford strongly suggestive evidence of the ability of the animal organism to convert glycolic acid to glycine to a small extent.

That there may be a species difference in the ability to convert glycolic acid to glycine is indicated in a recent note by Almquist and Mecchi (14). They found that glycolic acid could not be utilized by the chick in place of glycine, a compound essential for the growth of chicks.

SUMMARY

N-Ethylglycine, in contrast to N-methylglycine (sarcosine), caused no marked increase in rate of excretion of hippuric acid when administered with benzoate to rabbits. This is interpreted as evidence that "deethylation" to yield glycine has not occurred readily as does demethylation of the corresponding N-methyl derivative.

Glycolic acid, when administered with benzoic acid (3 moles per mole of benzoate), caused some increase in the rate of excretion of hippuric acid, an increase which was, however, distinctly less than that observed after administration of sarcosine or glycine. This increase in rate of excretion of hippuric acid was interpreted as evidence of the ability of the rabbit to convert glycolic acid to glycine to a limited extent.

[2] Dr. Abraham White of Yale University has stated privately to us that, "The superimposition of glycine, sarcosine, or glycolic acid, in experimentally determined *optimal* quantities, on a benzoate-containing basal diet used to inhibit the growth of young rats, results in a prompt increase in growth rate; the growth-promoting effect of glycolic acid is less striking than that of either glycine or sarcosine."

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A NOTE ON THE PURIFICATION OF LECITHIN

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The purification of lecithin is usually accomplished by precipitation with cadmium chloride and washing the cadmium salt with ether, according to the directions of Levene and Rolf (1). The method is somewhat tedious, since more than one precipitation with cadmium is usually necessary. It seems worth while, therefore, to describe a modified procedure for purifying the cadmium salt, which offers some advantages both in convenience and in the purity of the final product; the yields are comparable to those obtained by other methods.

The cadmium salt of lecithin is not soluble in petroleum ether and only slightly soluble in 80 per cent ethyl alcohol; it is, however, quite readily soluble in 80 per cent alcohol saturated with petroleum ether, and separates from the solution when the petroleum is removed. This makes possible a new procedure of combined extraction and crystallization, as follows: The phosphatides are precipitated from alcoholic solution with cadmium chloride. The precipitate is washed with alcohol; then, either with or without being first washed with ether, it is suspended in petroleum ether with from 150 to 200 ml. for each 10 gm. of phosphatide. The mixture is repeatedly extracted with from one-fifth to one-fourth its volume of 80 per cent alcohol, petroleum ether being added from time to time to keep the volume constant. Ten to twelve extractions may be necessary, but, since the solvent layers separate readily with no tendency to emulsify, the whole process may be completed rapidly. The successive extracts may be tested by taking small samples and removing the petroleum ether by aeration, when a white precipitate readily separates. As soon as this test gives only a scant, difficultly coagulable precipitate, the extraction should be discontinued.

The alcoholic solution is next freed from petroleum ether *in vacuo* at about 35° and allowed to stand overnight in the cold before the precipitate is collected. If further purification appears necessary, it may be accomplished by taking up the precipitate in petroleum ether and extracting with 80 per cent alcohol as before. The cadmium compound so purified gives a perfectly clear solution in chloroform, in contrast to material purified only by washing with ether, which is often contaminated with chloroform-insoluble impurities.

The results of some typical preparations are given below. It is apparent that satisfactory preparations of lecithin are easily obtained by this method, even when one starts with material badly contaminated by decomposition products, a point of some practical importance if commercial lecithin is to be used. In the experiments reported, preliminary washing with ether was used in order to compare the effectiveness of the two methods. It was found that cadmium salts repeatedly washed with ether retained appreciable amounts of ether-soluble impurities that could be removed by the petroleum ether-alcohol method without the necessity of a second cadmium precipitation. On the other hand, by this method sphingomyelin and similar impurities are not removed, but must be separated by treatment with cold ether in the usual way; nor is plasmalogen removed (2). All lecithin preparations from beef heart gave strongly positive tests for plasmalogen by the technique of Feulgen, Imhäuser, and Behrens (3), no matter what method of purification was used.

EXPERIMENTAL

*Lecithin from Beef Heart—*An alcoholic extract of acetone-dried beef heart tissue was precipitated with cadmium chloride and the precipitate washed first with alcohol, then with ether, until the washings were nearly colorless, and once more with alcohol to remove ether. It was then suspended in petroleum ether (b.p. 30–60°), extracted thirteen times with 80 per cent alcohol, and the cadmium salt recovered from the extract as described above. The purified salt was dissolved in chloroform, and the cadmium was removed with methyl alcoholic ammonia (1). The chloroform solution was shaken with 10 per cent sodium chloride, and 1 N hydrochloric acid added cautiously until the aqueous phase

remained just acid to litmus after vigorous shaking. This step has been found convenient to insure the removal of excess ammonia. The chloroform solution was washed by shaking with 10 per cent sodium chloride to remove any excess hydrochloric acid; when necessary to break emulsions alcohol was added and the solution centrifuged. The lecithin recovered by evaporation of the chloroform contained an ether-insoluble impurity and was therefore further purified by repeated chilling of the ether solution and centrifugation in the cold. The yield of purified lecithin from 10 kilos of fresh tissue was 10.9 gm. A 10 per cent solution of this material in absolute alcohol was completely colorless.

Analysis—N 1.91, P 4.00, NH$_2$-N 0.00, iodine number 68.8

The material remaining in the petroleum ether after the lecithin salt was extracted was also freed from cadmium and recovered. This substance, Fraction P, weighed 0.9 gm. and had N 1.67, P 3.86, and NH$_2$-N 0.37.

As a check on the completeness of the purification, a portion of the lecithin, 9.6 gm., was reprecipitated with cadmium chloride and the petroleum ether-alcohol separation carried through as before. Fraction P in this case weighed only 87 mg. The repurified lecithin, 6.6 gm., had N 1.91, P 3.97, and NH$_2$-N 0.00. It is apparent that a satisfactory degree of purity had been attained by the first treatment.

Commercial Lecithin from Eggs—A sample of Merck's egg lecithin, 28 gm., was dissolved in absolute alcohol and freed from a little insoluble material. The dark brown alcohol solution was precipitated with cadmium chloride; the precipitate was washed first with alcohol, then seven times with ether, and, finally, with alcohol. It was suspended in 400 ml. of petroleum ether and extracted eleven times with 75 ml. portions of 80 per cent alcohol. The material remaining in the petroleum layer was freed from cadmium; it weighed 1.9 gm. and gave a dark brown solution in alcohol. The analysis showed N 1.64, P 2.97, NH$_2$-N 0.16, and iodine number 58.6.

The 80 per cent alcohol extract was freed from petroleum ether, chilled, and the nearly white precipitate separated and once more purified by the petroleum ether-alcohol method. The second treatment removed 0.2 gm. of petroleum ether-soluble

material. The recovered lecithin was nearly colorless, completely soluble in cold ether or absolute alcohol; it had N 1.81, P 3.82, NH_2-N 0.00, and iodine number 52.2; it weighed 6.6 gm. A second crop, 4 gm., was obtained by concentrating the 80 per cent alcohol filtrate *in vacuo* to about one-fourth its volume and placing the solution in a refrigerator for 3 days. The precipitate that separated was freed from cadmium without further purification. The recovered lecithin was pale yellow. It had N 1.86, P 3.95, NH_2-N 0.06, and iodine number 56.7.

For the analyses reported in this paper the author is indebted to Mr. Leonard W. Hyman of this laboratory.

SUMMARY

An improved method for purifying the cadmium salt of lecithin is described and some of its advantages and limitations are discussed.

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THE SYNTHESIS OF RADIOACTIVE LACTIC ACID

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The discovery of radioactive carbon, C^{11}, has made available a new tool for research in organic and physiological chemistry but the short half life of the isotope (only 20 minutes) places severe limitations on its application. In only a few instances has chemical use been made of this radioactive tracer (1). The following is an account of procedures which have been developed for the synthesis of two radioactive lactic acids. In one lactic acid the radioactive carbon is in the carboxyl position. In the other the radioactive carbon is divided between the carbon atoms α and β to the carboxyl group.

$CH_3CHOHC^{11}OOH$ $CH_3C^{11}HOHCOOH$ and $C^{11}H_3CHOHCOOH$
Radioactive lactic acid, Type I   Radioactive lactic acid, Type II

Synthesis of Radioactive Lactic Acid of Type I—This synthesis may be of general usefulness in work with radioactive carbon because it involves the preparation of radioactive potassium cyanide as an intermediate from which, by several standard methods, a variety of organic compounds may be obtained.

Radioactive carbon is ordinarily obtained by the bombardment of boron or boron oxide with deuterons. It is rather doubtful whether one could obtain elementary carbon from such bombardment and hence in the synthesis one should consider mainly boron carbide or the oxides of carbon (2) as starting material. In an attempt to use the former, the reaction between carbon and ammonia (3) resulting in the formation of ammonium cyanide has been reinvestigated and found to occur rapidly and efficiently, but attempts to induce it on heating boron carbide with ammonia failed. Even mixtures of a small quantity of free carbon with boron carbide failed to react under conditions of temperature

at which carbon alone was quantitatively converted into ammonium cyanide. Next the reaction between carbon dioxide and ammonia was tried, but, contrary to patent claims (4), no reaction was found to take place in 10 minutes, either homogeneously or on porcelain or catalytic iron and platinum surfaces at temperatures up to 1000°. Addition of hydrogen did not alter the negative results obtained in the previous experiments, but when carbon dioxide was replaced by carbon monoxide, small yields (1 to 2 per cent calculated on carbon) of cyanide were obtained.

According to Moissan (5) most of the carbon in the system carbon-magnesium-magnesium carbide is in the free form above 874°. It was hoped that magnesium would reduce carbon dioxide to carbon which, in turn, could be made to react with ammonia. These experiments, however, failed also, magnesium carbide, oxide, and nitride being the products and no cyanide being detected. To reconcile these results with the work of Moissan and others, one must conclude that magnesium cyanide reacts rapidly with excess metallic magnesium, forming the compounds observed.

The reaction by which cyanide was finally synthesized in a very satisfactory manner has been described by Delbrück (6).

$$4K + NH_3 + CO_2 = 2KOH + KH + KCN$$
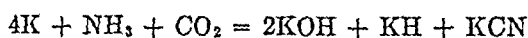
The reaction was carried out in a sealed tube, potassium being freshly distilled into the tube in a vacuum. With about 10^{-3} mole of carbon dioxide it was found that better yields were obtained with about 1 gm. of potassium metal than with 0.5 or 0.25 gm., but further increase to 2 gm. did not improve the results significantly; excess of ammonia (2:1) slightly favored the yield calculated on carbon, as much as 50 to 60 per cent being converted to cyanide in 10 minutes at 525°. Longer heating was definitely detrimental to the yields and lower temperature did not improve them either. A similar reaction in which potassium was replaced by sodium was found to be less satisfactory.

In the over-all procedure which was finally adopted, boron oxide was bombarded by deuterons in the Harvard cyclotron and the oxide was scraped from the copper target and placed in an iron boat on the bottom of which was spread in a thin layer sufficient calcium carbonate to give about 10^{-3} mole of carbon dioxide. It is necessary thus to dilute the radioactive carbon dioxide in

order to have sufficient material to manipulate, since less than 10^{-12} per cent of the lactic acid molecules synthesized contains a radioactive carbon atom. The boat was pushed into an electrically heated furnace at about 600° and as the oxide melted it reacted vigorously with the calcium carbonate. The escaping gas bubbles passed through the molten mass and effectively removed the radioactive carbon oxides. The gases, carried by a slow stream of nitrogen, passed through a layer of heated copper oxide and then into a liquid air-cooled trap in which carbon dioxide was condensed. The trap was disengaged from the furnace and evacuated while still being chilled in liquid air. In this manner any nitrogen which had condensed in the trap was removed but the carbon dioxide remained. The carbon dioxide was then distilled from the trap into an attached tube in which potassium and a measured amount of ammonia had been condensed beforehand. The tube was sealed off and placed in an electric furnace heated to 525° for 10 minutes, whereupon the formation of potassium cyanide took place. After removal from the furnace and rapid cooling, the excess potassium was destroyed by a cautious addition of water. About 1 cc. of acetaldehyde was added to the solution of potassium cyanide which had a volume of about 7 cc. and contained approximately 6×10^{-4} mole of cyanide ion. The reaction of cyanide with acetaldehyde is almost quantitative under these conditions and is very rapid because of the free alkali in the solution (7). An excess of acetaldehyde is needed, in view of the reversible nature of the reaction (8).

The hydrolysis of nitriles to carboxylic acids is catalyzed by mineral acids, particularly hydrochloric acid. From previous work on the hydrolysis of various nitriles (9) it was evident that in order to complete the hydrolysis of α-hydroxypropionitrile rapidly extreme conditions were needed. The alkaline solution of the nitrile was neutralized by addition of concentrated hydrochloric acid and then more was added so that the final acid concentration of the solution was approximately 8 M. The tube was sealed off and heated at 100°. Under these conditions hydrolysis is complete within 5 minutes.

Considerable difficulty was encountered in attempts to separate the lactic acid thus produced from the large excess of inorganic ions and from the polymerization products of the excess acetalde-

hyde. Extensive experimentation with heavy metal salts of lactic acid (10) revealed that the basic stannous salt was most favorable from the point of view of its solubility and stability to hydrolysis. However, it was found that, contrary to information in the literature, this salt could not be precipitated from solutions to which acid was added and only part of the lactic acid could be recovered in the reaction of tin salts with sodium lactate. The optimum conditions were obtained when slightly less than 1 mole of alkali was added to a mixture of 1 mole of sodium lactate and 1 mole of a stannous salt, but even then the yields were quite unsatisfactory. The method was finally abandoned because of poor yields and the excessive time required to obtain pure acid from the precipitated tin salt. Several methods were tried next which were all based on different solubilities of various lactic acid salts in non-aqueous solvents but were also rejected as unsatisfactory. Extraction of lactic acid from its aqueous solution with organic solvents was also abandoned because of the time required to concentrate lactic acid and to free it from organic impurities.

The procedure finally adopted consisted in neutralizing the acid hydrolysate solution with sodium hydroxide and evaporating it to dryness by warming it on a steam bath under the vacuum of a water pump. The residue of dry salts was then made just acid to phenol red indicator by the addition of a solution of hydrogen chloride in diethyl ether and the lactic acid liberated was extracted from the inorganic salts with diethyl ether. Five 75 cc. portions of ether were used. The combined ether extracts were concentrated to a total volume of 10 cc. and the lactic acid was extracted from the ether solution with several 5 cc. portions of water. The water extract contained around 50 mg. of lactic acid.

The lactic acid was used in metabolism studies and for this purpose it was desirable to have a sample containing approximately 150 mg. of lactic acid. This sample was prepared by adding 100 mg. of ordinary lactic acid to the 50 mg. sample containing radioactive acid and diluting the whole to exactly 25 cc. Several 1 cc. samples were withdrawn by pipette for the determination of the exact lactate content and for use as a standard in the measurement of radioactivity. The remainder of the solution was exactly neutralized to the phenol red end-point with sodium hydroxide and evaporated to about 2 cc. volume. The product contained varying small amounts of sodium chloride.

Summed up, the synthesis was carried out through the following steps,

$$C^{11}O_2 + K + NH_3 \rightarrow KC^{11}N$$
$$KC^{11}N + CH_3CHO \rightarrow CH_3CHOHC^{11}N$$
$$CH_3CHOHC^{11}N + H_2O \rightarrow CH_3CHOHC^{11}OOH$$
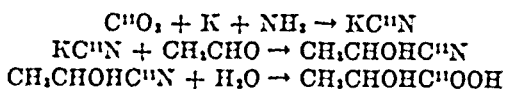
and required from 45 to 60 minutes. The subsequent purification and preparation for biological experiments required another 45 to 60 minutes and at the end some 30 to 40 per cent of the original radioactive carbon was present in lactic acid, if allowance is made for the disintegration which occurred meanwhile.

Synthesis of Radioactive Lactic Acid of Type II—This synthesis results in the formation of a product which is probably a nearly equimolecular mixture of $CH_3C^{11}HOHCOOH$ and $C^{11}H_3CHOH\text{-}COOH$. It consists of the following steps,

$$\left.\begin{array}{l} Na_2CO_3 + BaCl_2 \rightarrow BaCO_3 + 2NaCl \\ Na_2C^{11}O_3 + BaCl_2 \rightarrow BaC^{11}O_3 + 2NaCl \end{array}\right\}$$
$$BaC^{11}O_3 + BaCO_3 + Mg \rightarrow BaCC^{11} + MgO.$$
$$BaCC^{11} + H_2O \rightarrow HC\equiv C^{11}H$$
$$\left.\begin{array}{l} HC\equiv C^{11}H + H_2O \rightarrow CH_3C^{11}HO \\ HC\equiv C^{11}H + H_2O \rightarrow C^{11}H_3CHO \end{array}\right\}$$
$$\left.\begin{array}{l} CH_3C^{11}HO + KCN \rightarrow CH_3C^{11}HOHCN \\ C^{11}H_3CHO + KCN \rightarrow C^{11}H_3CHOHCN \end{array}\right\}$$
$$\left.\begin{array}{l} CH_3C^{11}HOHCN + H_2O \rightarrow CH_3C^{11}HOHCOOH \\ C^{11}H_3CHOHCN + H_2O \rightarrow C^{11}H_3CHOHCOOH \end{array}\right\}$$
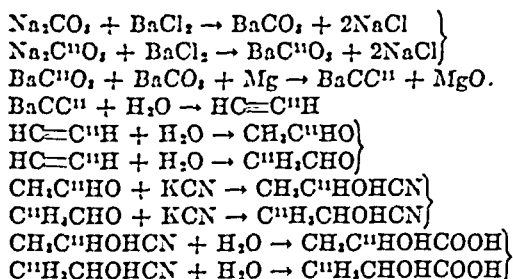
The radioactive oxides of carbon were prepared as described above. They were swept in a current of nitrogen through the heated copper oxide in the combustion tube into a trap which was chilled in liquid air. The trap contained 10 cc. of an approximately 2 M frozen solution of sodium hydroxide. After the boron oxide had been fused for 15 minutes, the trap was disengaged from the combustion tube and evacuated to remove any condensed nitrogen. It was then warmed to room temperature, whereupon the sodium hydroxide solution melted and absorbed the carbon dioxide.

The solution of sodium hydroxide and sodium carbonate was rinsed into 10 cc. of an approximately 1 M solution of barium chloride. The barium carbonate which precipitated was filtered off and washed with water, alcohol, and ether. Maquenne (11) has investigated the reaction of barium carbonate, magnesium, and carbon in which barium carbide is formed as a source of acetylene. In order to avoid further dilution of the C^{11} with ordinary carbon,

barium carbonate and magnesium were heated together in an atmosphere of hydrogen at around 700°. It was found that under these conditions 60 per cent or more of the carbon in barium carbonate could be converted through barium carbide into acetylene.

The barium carbonate which contained some radioactive carbon was mixed intimately with 1 gm. of magnesium filings and the mixture was placed in an iron boat. The boat was shoved into an iron tube which was heated to about 700° by an electric furnace and through which passed a slow current of hydrogen. After 5 minutes the iron tube was removed from the furnace and was rapidly cooled in a spray of water. The boat was removed from the tube and placed in a test-tube. By the addition of water and gentle warming acetylene was formed from the barium carbide. This acetylene was carried in a current of nitrogen into a trap which was chilled in liquid air. The reaction of barium carbide with water appears to be less vigorous than that of calcium carbide and about 10 minutes were required to drive off all the acetylene. The trap containing acetylene was evacuated to remove any condensed nitrogen and the acetylene was distilled into a tube which contained 10 cc. of a catalyst solution for the hydrolysis of acetylene to acetaldehyde.

The hydrolysis of acetylene has been subjected to considerable investigation. The catalyst solution described by Scharf (12) which may be prepared by dissolving 2 gm. of mercuric sulfate and 6 gm. of sulfuric acid in water and diluting to 100 cc. was found to be quite satisfactory. At 100° it will hydrate 75 per cent or more of a sample of acetylene to acetaldehyde within 5 minutes.

The tube containing acetylene and the mercuric sulfate-sulfuric acid solution was sealed off and heated to 100° for 5 minutes. The tube was opened and the contents were rinsed into a flask. The acetaldehyde was distilled in a current of air into 5 cc. of a 2 M solution of potassium cyanide. The solution of α-hydroxypropionitrile and potassium cyanide was transferred to a glass tube. Sufficient concentrated hydrochloric acid was added to make the resulting solution about 8 M with respect to hydrogen chloride and the tube was sealed and heated to 100° for 5 minutes. The lactic acid formed was purified and prepared for biological experiments in the manner that has been described in the synthesis of lactic acid of Type I. The synthesis of the acid required about

105 minutes and purification and preparation for biological experiments required 45 minutes more. At the end of the synthesis some 40 to 50 per cent of the original radioactive carbon was present in lactic acid, allowance being made for the disintegration which occurred meanwhile.

It is a pleasant duty for the authors to thank Dr. James B. Conant for suggesting this problem. Thanks are also due Dr. A. K. Solomon and the Harvard cyclotron group who prepared the activated boron oxide targets.

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METABOLISM OF LACTIC ACID CONTAINING RADIO-ACTIVE CARBOXYL CARBON

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Although many steps concerned in the breakdown of carbohydrate are now quite well understood, the steps involved in the synthesis of glycogen from simple 3-carbon compounds are still obscure. The carbon of the glycogen formed following the administration of lactate to an animal may conceivably come from (a) the 3 carbon atoms of the administered lactate, (b) a 2-carbon residue left after decarboxylation of the lactate molecule (1), (c) other glycogen precursors already present or formed in the organism, (d) carbon dioxide produced by metabolic activity. We have used radioactive carbon (C^{11}) in an attempt to provide further information on the source or sources of glycogen carbon.

Since the half life of C^{11} is short (20.6 minutes), experiments were necessarily performed which could be completed within 6 to 7 hours. For this purpose, a rapid synthesis of lactic acid, containing C^{11} in the carboxyl position (Type I), was devised (2). Rats were then fed this material as sodium lactate; and, after a lapse of 2.5 hours, the liver glycogen was isolated and its content of radioactive carbon determined.

If the newly formed glycogen contained radioactivity *in the same proportion* as it existed in the administered lactate, one might conclude that the glycogen did indeed arise from the lactate administered (source (a)). If the proportion of radioactivity were significantly less, the alternative sources (b) and (c) must also be

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considered. The data to be subsequently presented indicate, in fact, that the carbon of the new liver glycogen does not have its origin solely in the 3 carbon atoms of the lactate molecules.

The preparation of the lactate solutions required about 2 hours after the sample of C^{11} was removed from the cyclotron. The radioactivity of these lactate preparations could be subsequently followed with an accuracy of 1 per cent for 3.5 to 4.5 hours. Consequently, 2.5 hours were allowed for the biological process, leaving 1 to 2 hours for the isolation of glycogen and the measurement of its radioactivity.

We first made a series of experiments with C^{12} lactate,[1] following the technique of Cori and Cori (3), to determine how closely we could reproduce their results within our restricted time interval. Hooded and albino rats weighing between 100 and 185 gm. before fasting were employed. The weights of the animals at the time of the experiment were recorded. The livers were removed under amytal anesthesia and dropped promptly into 30 per cent KOH. The procedure of Good, Kramer, and Somogyi (4) was used for glycogen determinations, except that hydrolysis was carried out in 5 N H_2SO_4 as suggested by Sjögren (5). Sugar was estimated by the method of Folin and Wu (6). Lactate determinations on the intestinal contents and on the urine were carried out by the method of Friedemann and Graeser (7) after preparation of the material according to the procedure of Cori and Cori (3).

Experiments with C^{12} Lactate—Liver glycogen was first determined in a series of controls fasted for 24 hours. These results are given in Table I, yielding an average figure of 0.12 mg. of glycogen per 100 mg. of liver, which is in good agreement with results reported by others. In four experiments, the lactate of the intestine and the urine was determined in order to provide control data for subsequent experiments. The intestine contained an average of 5 mg. of lactate, and the urine, 0.5 mg.

A second series of fasted rats was fed C^{12} lactate by stomach tube. In these, as well as in the radioactive lactate experiments, racemic lactate was used, administered as the sodium salt dis-

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[1] C^{12} lactate will subsequently be used in this paper to differentiate experiments with ordinary *dl*-lactate from those with radioactive *dl*-lactate, which will be designated C^{11} lactate.

solved in 2 cc. of water. Table II shows that, based on the lactate administered as 100, one might conclude that 31 per cent had

TABLE I

Liver Glycogen of Control Rats, Fasted 24 Hours

Rat weight	Liver		Rat weight	Liver	
	Weight	Glycogen*		Weight	Glycogen*
gm.	gm.	per cent	gm.	gm.	per cent
90	3.51	0.02	161	5.49	0.02
102	3.66	0.47	145	5.02	0.52
98	3.50	0.09	136	5.25	0.14
90	3.35	0.01	118	4.40	0.06
89	3.34	0.02	112	4.91	0.09
119	4.24	0.04	135	4.89	0.05
117	3.92	0.03	139	5.05	0.07
Average					0.12

* Expressed as mg. of glucose per 100 mg. of wet liver.

TABLE II

Liver Glycogen of Fasted Rats, 2.5 Hours after Feeding C^{11} Lactate

Rat weight	Lactate			Liver		Glycogen	
	Fed	Absorbed	Urine	Weight	Glycogen	Formed*	Per cent of lactate fed
gm.	mg.	mg.	mg.	gm.	per cent	mg.	
91	150	117	1.9	3.92	1.72	63	42
93	150	123	2.0	3.67	2.03	70	47
100	150	95	4.6	3.57	1.00	31	21
97	150			3.59	0.94	29	19
134	161	159		5.14	0.76 *	33	20
117	109	103		4.43	0.97	38	35
148	200	141		5.18	0.79.	35	18
125	200	143		4.06	0.32.	8	4
132	200	159		5.12	1.47	69	35
126	200	169		5.36	1.95	98	49
131	96	77		5.41	1.06	51	53
Average					1.18		31

* Corrected for amount found in livers of fasted controls (0.12 per cent).

appeared as glycogen. The average glycogen content, 1.18 per cent, is well above 0.12 found for the fasted controls. These

results are consistent with those of Cori and Cori (3), and show that feeding racemic C^{12} lactate to our rats produces within 2.5 hours sufficiently large increases in liver glycogen to justify the study of the presence or absence of radioactivity (Table II).

Experiments with Radioactive Lactate—A similar series of experiments was then made with C^{11} lactate ($CH_3CHOHC^{11}OONa$). The glycogen was quantitatively isolated for the determination of its amount and of its radioactivity, and the amount of unabsorbed lactate was determined chemically. In addition, the CO_2 expired during the 2.5 hour period was collected to determine the amount of C^{11} excreted by this path. The procedure used will be briefly described.

After the rat had been fed, it was placed in a glass jar furnished with inlet and outlet tubes. An air current was drawn briskly from the jar through a sintered glass plate into a column of dilute KOH which absorbed the CO_2. This solution was changed each half hour. The carbonate was precipitated as $BaCO_3$ with $Ba(NO_3)_2$, filtered by suction, washed with water, and dried with alcohol and ether before measurement of its weight and radioactivity.

After removal of the liver, the glycogen was isolated as rapidly as possible. An aliquot of the alkaline hydrolysate was taken for chemical determination, and the remainder was precipitated with alcohol. Insoluble impurities were removed by centrifugation from the first water solution; then the second precipitation was carried out at a slightly acid pH (approximately pH 4) to prevent the precipitation of carbonates. The glycogen was filtered off, washed with alcohol, and dried with ether before its radioactivity was determined. In some cases, enough time was available so that the activity could be redetermined after another precipitation. There was no evidence that this procedure changed the C^{11} content of the preparation. For determination of its radioactivity, the urine was made basic and evaporated to dryness.

The amount of lactate fed was determined chemically on an aliquot and corrected for loss in transfers.

Radioactive Technique—An alcohol-quenched Geiger-Müller counter was used for measurement of the positrons from C^{11}. The counts were recorded by a Cenco impulse counter connected to a scale of 16 circuit (8). Since the positrons given off by C^{11}

produce annihilation γ-rays, the counter and sample were shielded by an inch of lead. The half life of C^{11} determined in these experiments is 20.63 ± 0.03 minutes.

Glycogen and an aliquot of the lactate solution were counted directly, the first as a precipitate, and the second after evaporation to dryness. Since the lactate samples, as well as the samples of glycogen, weighed but a few mg., no correction was required for self-absorption of the radiation. However, the carbonate samples usually weighed about 600 mg. and self-absorption corrections were necessary. Extrapolation of the absorption curve of carbonate samples to zero carbonate weight gave a figure of 0.69 for the ratio of the activity of a sample weighing 600 mg. to the activity of a sample weighing 0 mg., in good agreement with direct conversion figures obtained by oxidation of radioactive lactic acid and dilution of the resulting CO_2 with inactive carbonate. Consequently, this figure was adopted in the calculations of the radioactivity of the expired CO_2. Due to the short half life of C^{11}, corrections were also made for decay of the samples during the counting.

Whenever time was available, 4800 counts were taken for each sample, giving a probable statistical error of 1 per cent. In some cases, including the glycogen counts, time was not available for such a long count, and estimates of the probable statistical error in the glycogen count are included in the tabulated results. Statistical error was usually less than random errors. Estimates of these random errors from figures obtained in the carbonate calibration experiments give a value of approximately 5 per cent for the average probable error.

Results with Radioactive Lactate—The results obtained with radioactive lactic acid are given in Tables III and IV. In three early experiments in which no chemical determinations were made, the amount of precipitated glycogen was always high. It may reasonably be assumed that the lactate absorbed and glycogen formed were comparable to those of the later experiments. Column 8 of Table III gives the glycogen formed as per cent of the lactate fed, and Column 9 gives the radioactivity as per cent of the radioactivity fed. In Column 11, the ratios of these two values are listed. Clearly, the results of these ten experiments show that

TABLE III

Radioactivity of Liver Glycogen 2.5 Hours after Feeding C^{11} Lactate

Experiment No. (1)	Rat weight (2)	Lactate Fed (3)	Lactate Absorbed (4)	Liver Weight (5)	Liver Glycogen (6)	Formed* (7)	Per cent of lactate fed (8)	Glycogen Radioactivity Per cent of amount fed (9)	Glycogen Radioactivity Error (10)	Ratio, Column 9 / Column 8 (11)
		gm.	mg.	mg.	gm.	per cent	mg.		per cent	
1	115	44						1.7	3	
2	115	30						<1.0	43	
3†	136	130						0.7	19	
4	108	150		4.30	1.72	69	46	2.1	5	0.05
5	100	50	50	3.60	0.77	23	46	2.4	18	0.05
6	111	109	104	4.63	1.88	81	74	1.8	21	0.02
7	122	214	81	4.44	1.09	43	20	1.3	13	0.06
8	140	196	117	5.24	0.46	18	9	3.1	3	0.34
9	140	250	171	5.33	0.67	29	12	1.4	5	0.12
10	121	162	126	4.65	0.72	28	17	0.7	3	0.04
Average							32	1.6		0.10

* Corrected for amount found in livers of fasted controls (0.12 per cent).
† 2 hour experiment.

TABLE IV

Excretion of C^{11} in Urine and Expired CO_2

Experiment No.	Radioactivity in expired CO_2 Per cent of amount fed 0.5 hr.	1.0 hr.	1.5 hrs.	2.0 hrs.	2.5 hrs.	Total per cent of amount fed	Total per cent of amount absorbed	Radioactivity in urine, per cent of amount fed
1	2.3	7.7	9.0	11.2	9.3	39.5		
2	1.2	4.6	6.8	5.2	4.2	22.0		3.5
3*	1.0	4.9	8.1	4.2		18.2		1.4
4	2.0	7.5	11.6	10.4	11.5	43.0		1.6
5	1.3	3.0	4.1	4.5	3.0	15.9	15.9	
6	0.6	1.2	4.1	3.9		>9.8	>10.3	
7	0.7	1.7	2.3	4.5	3.2	12.4	32.8	
8	0.6	1.9	3.0	3.5	4.5	13.5	22.6	
9	0.7	1.7	4.2	2.6	>1.2	>10.4	>15.2	
10	0.4	1.2	3.3	3.6	>2.5	>11.0	>14.1	
Average	1.1	3.5	5.6	5.4	4.9	20		

* 2 hour experiment.

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the carbon of the liver glycogen could not have originated entirely from the 3 carbon atoms of the lactate fed, because, in such a case, the ratios in Column 11 would have been unity. Although there is considerable variation, the ratio never approaches this value.

Table IV gives the percentage of radioactivity excreted in the urine and in the expired CO_2. Since the urine figures were so low, determinations were only made in a few runs. It is possible to account for some radioactivity by the presence of unchanged lactate in the body fluids; but this, according to Cori's data, should not exceed 15 mg. Furthermore, in runs in which the lactate fed amounted to only 50 mg., such a figure is certainly far too high. Consequently, we feel justified in assigning a generous maximum of 20 per cent for unchanged lactate present. Even when the radioactivity of this amount of lactate is added, it is clear that a large proportion of the radioactivity in each experiment is not accounted for.

DISCUSSION

The question may arise as to whether the radioactivity of the carbon, fed the rat, could cause any change in its metabolism. The animals showed no outward symptoms which could be attributed to the administration of a radioactive compound. Furthermore, the amount of glycogen formed in our radioactive experiments is in good agreement with the amount formed by rats fed ordinary C^{12} lactate. The total integral dose of radioactivity given the rat never exceeded 65 microcurie hours, which is not regarded as excessive.

Expired CO_2—The promptness with which radioactivity is found in the expired CO_2 indicates that the lactate was readily absorbed and, to some extent, metabolized within the first half hour after the administration of the lactate. The rate of excretion of radioactive CO_2 increased during the second and third half hour periods to an average of about 5 per cent of the administered radioactivity per half hour period. Radioactivity was still appearing in the expired CO_2 at approximately this rate when the animals were sacrificed at the end of 2.5 hours. However, considerable variation was found among the experiments, some animals showing maximum $C^{11}O_2$ excretion rates during the third half hour, others

during the fourth half hour. This is probably to be attributed
to the different rates at which the lactate was absorbed in different
animals.

The total CO_2 expired during the 2.5 hour period amounted on
the average to 12.5 mM. (This compares favorably with the value
12.7 mM calculated from Donaldson's figures (9) for the CO_2
excretion of the rat.) It is of interest to estimate what propor-
tion of this CO_2 could have arisen from the lactate fed. Since
approximately 20 per cent of the administered radioactivity ap-
peared in the expired CO_2, and since the lactate administered
amounted to approximately 1.5 mM, the expired CO_2 represented
the metabolism of 0.3 mM of lactate. The maximum and mini-
mum amounts of CO_2 obtainable from 1 mM of lactate would be
3 mM and 1 mM respectively. The amount of expired CO_2 arising
from the lactate administered would, therefore, have the maximum
value of 0.9 mM, or the minimum value of 0.3 mM. In either case,
it would represent but a small fraction of the total CO_2 expired
during the experimental period.

Interpretation of Glycogen Results—It is not possible with the
data at hand to state definitely the sources of the carbon of the
liver glycogen. However, as a guide to further investigation, we
may examine the results to see what sources of the glycogen carbon
have been eliminated and what ones have not.

1. The amount of radioactivity found in the glycogen was only
1.6 per cent of the administered radioactivity; whereas, the total
glycogen found could be said, from the chemical data alone, to
represent 30 per cent of the administered lactate. If the incom-
pleteness of absorption and the fact that only the absorbed
$l(+)$-lactate would lead to glycogen synthesis (3) are taken into
account, the per cent of available radioactivity appearing in the
glycogen would amount to 4 per cent. This fact seems to elimi-
nate the unbroken 3-carbon chain of the absorbed lactate molecules
as the principal source of the glycogen. One point to be borne in
mind, however, is that there exists a recognized interrelation
among the lactate, pyruvate, certain amino acids, and glucose
already or potentially present in the organism. Thus, the concen-
tration of absorbed radioactive lactate molecules may be reduced
by dilution in this "family" of glycogen-forming compounds.
The possible interchange of muscle and liver glycogen must also

be considered. How significant such interchange and dilution factors are in the present experiments cannot now be evaluated.

2. On the other hand, does the appearance of a small but definite amount of radioactive C^{11} necessarily mean that some of the glycogen did come from the original 3-carbon chain of the absorbed $l(+)$-lactate? Even this is not necessarily the case. Preliminary experiments, to be reported in detail later, have shown that when non-radioactive dl-lactate is administered to a rat together with radioactive CO_2 in the form of $NaHC^{11}O_3$, about 1 per cent of the administered radioactivity is to be found in the liver glycogen. Ruben and Kamen and their coworkers (10) have already demonstrated that a number of biological systems are capable of reducing $C^{11}O_2$. In view of these observations, it is possible that the presence of radioactivity in the glycogen indicates that its synthesis involves reactions in which the enzymatic addition and subsequent reduction of CO_2 play a prominent rôle.

SUMMARY

1. Rats have been fed solutions of lactate whose carboxyl carbon is radioactive, and the radioactivity of the expired CO_2 and liver glycogen measured.

2. The CO_2 expired during 2.5 hours following the feeding of the lactate accounts for about 20 per cent of the administered radioactivity.

3. Although the amount of liver glycogen formed, in terms of the lactate fed, would appear to account for 32 per cent of the lactate, the radioactivity of the glycogen accounts for only 1.6 per cent of the administered radioactivity.

4. In the light of these results, the sources of the carbon of liver glycogen are reexamined.

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POLAROGRAPHIC DETERMINATION OF RIBOFLAVIN (VITAMIN B₂) AND OTHER VITAMIN B FACTORS

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Colorimetric, fluorometric, and microbiological methods have been used for the determination of riboflavin. With the exception of an early study of nicotinic acid by Shikata and Tachi (7), no studies have been published concerning the polarographic behavior of riboflavin or any of the other vitamin B factors. Consideration of the structures of the various vitamin B complexes that have been isolated (thiamine, riboflavin, pyridoxine, nicotinic acid, and pantothenic acid) led us to believe that they should be reducible at the dropping mercury electrode, and hence amenable to polarographic determination. This expectation has been confirmed, and results obtained so far indicate that the polarographic method may prove to be of considerable value for the determination of the vitamin B factors.

Since this method promises to be particularly valuable for the determination of riboflavin in the presence of the other members of this group, we present at this time some preliminary results concerning the polarographic behavior of the various pure vitamin B complexes. Our studies have not yet been extended to the determination of these factors in natural products, although we do have evidence that the determination of riboflavin in such substances by the polarographic technique is entirely feasible.

The usual polarographic technique has been employed (4). The polarographic waves of the pure vitamins, in concentrations from about 5×10^{-6} up to 0.001 M, were recorded automatically with a Heyrovsky-Shikata type polarograph (2, 4). In general, well buffered solutions were employed, although for special reasons some experiments were made in unbuffered solutions.

Riboflavin—We have found that riboflavin gives good polaro-

graphic waves in practically any buffered or unbuffered solution, over a range of pH values from 4 to 9. However, the vitamin decomposes in alkaline solutions, and the optimum pH for its determination is in the neighborhood of 7.
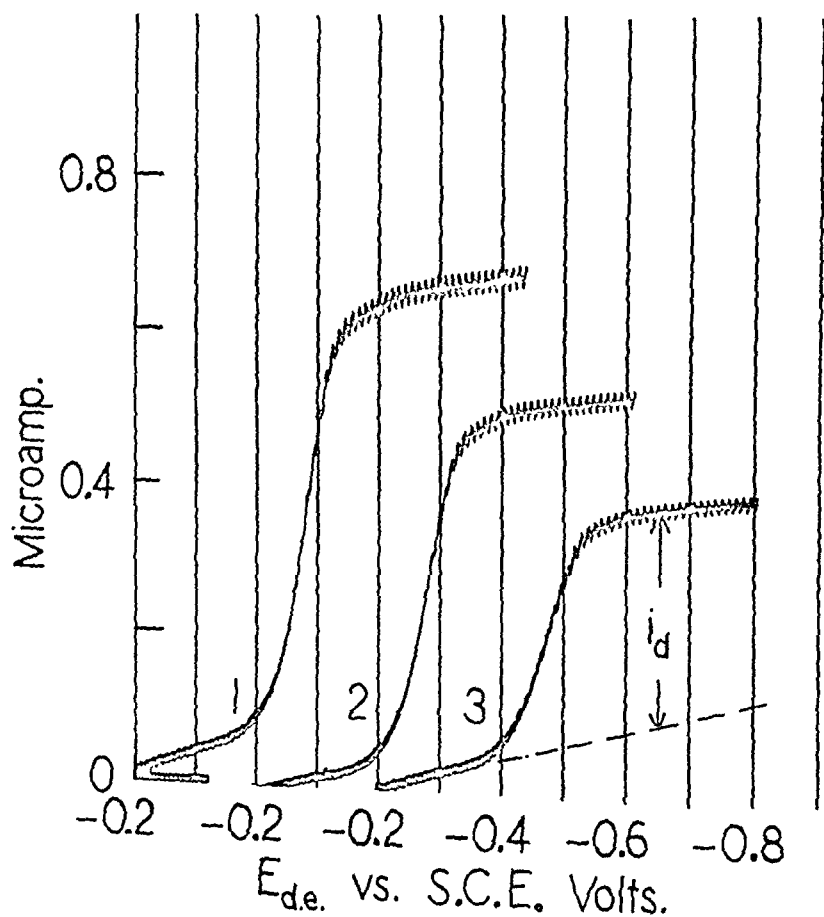
Fig. 1. Polarograms of riboflavin in a 0.1 M phosphate buffer of pH 7.2. The concentration of riboflavin for Curve 1 was 0.133, for Curve 2, 0.106, and for Curve 3, 0.0798 mM per liter, corresponding to 50, 40, and 30 p.p.m.

A typical set of curves of three different concentrations of riboflavin in a 0.1 M phosphate buffer of pH 7.2 is shown in Fig. 1. In this and all subsequent polarograms, the potential of the dropping electrode, $E_{d.e.}$, is referred to a saturated calomel reference electrode. Due to the small concentrations of riboflavin that were used, and the corresponding large galvanometer sensitivity,

the slope of the "residual current" (4) preceding the waves is quite pronounced. However, the diffusion current, i_d, is easily measurable by the method indicated.

In general, the true diffusion current of a substance is equal to the difference between the *total* current and the residual current of the supporting electrolyte alone (4, 5). The residual current can be determined in a separate experiment without any of the reducible substance present. However, when the residual current is practically a linear function of $E_{d.e.}$, as it is in the present case, the extrapolation method of measuring i_d shown in Fig. 1 is quite

TABLE I

Linear Relation between Concentration of Riboflavin and Its Diffusion Current in Tetramethylammonium Phosphate Buffer of pH 7.2

Riboflavin concentration		i_d	i_d/C
p.p.m.	mM per l.	microampere	microamperes per mM per l.
2	0.0053	0.023	(4.3)
4	0.0106	0.040	3.8
5	0.0133	0.053	4.0
10	0.0266	0.102	3.84
20	0.0532	0.203	3.82
30	0.0798	0.300	3.76
40	0.106	0.403	3.78
50	0.133	0.514	3.86
Average of last 7..			3.84 ± 0.05

satisfactory. The various factors which govern polarographic diffusion currents have been discussed in detail by Ilkovic (3), and Kolthoff and Lingane (4, 5).

The diffusion current obtained with a given capillary, and with all other conditions constant, is directly proportional to the concentration of the reducible substance (3–5). We have tested this relation in the case of riboflavin, and obtained the results shown in Table I. These data show that i_d is directly proportional to the concentration of riboflavin, even down to a concentration as small as 5×10^{-6} M (2 p.p.m.). The diffusion current, and hence the concentration of riboflavin, is measurable with a precision of about ± 3 per cent over the concentration range from 10^{-5}

to 10^{-4} M (4 to 50 p.p.m.). The minimum determinable concentration is about 5×10^{-6} M (2 p.p.m.), but somewhat smaller concentrations can be detected.

The diffusion current constant of riboflavin (i_d/C) depends somewhat on the pH and type of buffer that is used, and hence calibration data for a given capillary must be obtained under the same conditions that prevail in an actual determination.

In general, when hydrogen or hydroxyl ions are involved in an electrode reaction, the half wave potential will depend on the pH of the buffer used as supporting electrolyte (1, 4, 6). The half wave potentials of the various vitamin B complexes in buffered

TABLE II

Half Wave Potentials of Vitamin B Factors in Various Media

The values given are the half wave potentials, in volts, referred to the saturated calomel electrode at 25°, in 0.1 M buffers of various pH; biphthalate buffer for pH 5.8, phosphate buffer for pH 7.2, and borate buffer for pH 9.

Substance	pH 5.8	pH 7.2	pH 9.0	0.1 N KCl
Riboflavin............	−0.41	−0.47	−0.54	−0.35
Thiamine.............	*	−1.3	†	−1.25
Nicotinic acid.........	*	−1.56	−1.6	−1.7
Pyridoxine............	*	*		−1.8, −2.0‡, §
Pantothenic acid.......	*	*		−2.0‡

* Hydrogen wave interferes.
† Vitamin unstable.
‡ In 0.1 M tetramethylammonium bromide.
§ Double wave (see Fig. 3).

solutions of different pH and in unbuffered potassium chloride and tetramethylammonium bromide solutions are shown in Table II. Since hydrogen ions are consumed in the reduction of these substances, the half wave potentials are shifted to a more negative value with increasing pH.

When a reaction of the type $R + n\,e + n\,H^+ \rightleftharpoons RH_n$ takes place reversibly at the dropping electrode, it can be shown that $E_{\frac{1}{2}}$ should shift by 0.059 volt to a more negative value per unit increase in the pH of the buffer (1, 4, 6). The observed shift of the half wave potential of riboflavin is somewhat less than the theoretical shift, especially at higher pH values. This may be due either to

the fact that the reduction of riboflavin is not perfectly reversible, or that its reduction product exists partly in the ionic state at a pH greater than 7. The interpretation of these data is further complicated by the fact that riboflavin decomposes slowly in alkaline solutions.

It will be noted that riboflavin is the most easily reducible of the vitamin B complexes, and hence its determination in the presence of the other B factors should present no particular difficulty as far as interference from the other members of the group is concerned.

Preliminary experiments have shown that the riboflavin content of dilute hydrochloric acid extracts of yeast can be determined by the polarographic technique, after the pH of the extract is adjusted to about 7 with sodium hydroxide.

Thiamine—Thiamine is reducible at the dropping electrode, but its polarographic determination is complicated by several factors. In a phosphate buffer of pH 7 a wave is obtained, with a half wave potential of −1.3 volts *versus* the saturated calomel reference electrode, but the diffusion current is not very well defined and is partly masked by the evolution of hydrogen from the buffer acid. Attempts were made to shift the half wave potential to a more positive value by using a buffer of lower pH. However, when the pH is decreased, the hydrogen wave shifts in the positive direction more rapidly than that of the thiamine, and in buffers of pH less than 7 the discharge of hydrogen from the buffer acid coincides with, or precedes, the reduction of thiamine and hence no wave for the latter can be observed. In buffers of pH greater than 7 thiamine produces a double wave, but the character of the wave changes with time, apparently because of the instability of thiamine in alkaline solutions. In 0.1 N sodium hydroxide no wave was obtained.

In unbuffered potassium chloride solutions a fairly good wave was obtained, with a half wave potential of −1.25 volts *versus* the saturated calomel reference electrode.

Nicotinic Acid—A well defined and easily measurable wave was obtained with nicotinic acid in a tetramethylammonium borate buffer of pH 9. The half wave potential was −1.6 volts *versus* the saturated calomel reference electrode.

In buffers of pH less than 7 the discharge of hydrogen masks the

wave of nicotinic acid. A fairly well defined wave was obtained in unbuffered solutions of potassium chloride, after the nicotinic acid was neutralized with sodium hydroxide. Buffered solutions of pH between 8 and 9 are most suitable for the determination of nicotinic acid.
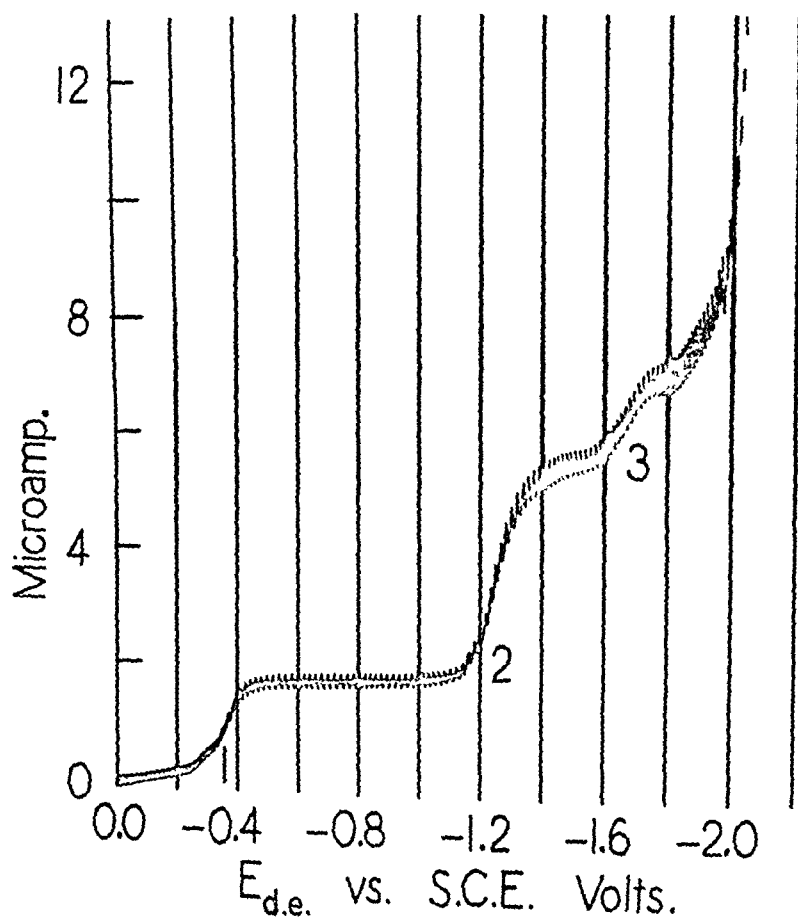
FIG. 2. Polarogram of a solution containing 3×10^{-4} M riboflavin (Wave 1), 4×10^{-4} M thiamine (Wave 2), and 4×10^{-4} M sodium nicotinate (Wave 3) in unbuffered 0.1 N potassium chloride.

Shikata and Tachi (7) obtained fairly good waves in 0.1 N sodium bicarbonate (pH about 8.4), with half wave potentials in the neighborhood of -1.6 volts *versus* the saturated calomel reference electrode. However, the half wave potential shifted somewhat to a more positive value with increasing concentration of nicotinic acid, indicating that the reduction is irreversible. Shikata and

sirable, is necessary in this particular case.   In buffered solutions of pH 7, or lower, the hydrogen wave interferes with the waves of thiamine and nicotinic acid, and at a pH greater than 7 the thiamine is unstable.

Pantothenic Acid and Pyridoxine—These substances were found to be quite difficult to reduce, although fairly satisfactory waves were obtained by using tetramethylammonium bromide as the supporting electrolyte, as shown in Fig. 3.   It was necessary to neutralize the pantothenic acid, and the pyridoxine hydrochloride, with tetramethylammonium hydroxide in order to prevent the appearance of an interfering hydrogen wave.

It will be noted that the pyridoxine produces a double wave, corresponding to reduction in two 2 electron steps.   These waves are being investigated further.

We express our appreciation to Mr. T. D. Sanford of the Research and Biological Laboratories, F. E. Booth and Company, Inc., for furnishing us with samples of the pure vitamin B factors.

SUMMARY

The polarographic behavior of riboflavin, thiamine, nicotinic acid, pantothenic acid, and pyridoxine was investigated.   All of these vitamin B complexes are reducible at the dropping mercury electrode, riboflavin being the most easily reducible of the group.

The diffusion current of riboflavin, in a phosphate buffer of pH 7.2, was found to be directly proportional to its concentration over the range from 5×10^{-6} to 10^{-4} M (2 to 50 p.p.m.).   Riboflavin can be determined in the presence of the other vitamin B factors, and its polarographic determination in natural products (*e.g.*, yeast) appears to be a practical possibility.

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ISOLATION AND PROPERTIES OF A PURE YEAST POLYPEPTIDASE*

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A yeast polypeptidase has been described by Grassmann and his collaborators (2, 3), who have obtained a highly active preparation of the enzyme (4). In the present investigation a yeast polypeptidase, not identical with the enzyme described by Grassmann, was isolated as an apparently homogeneous, highly active protein.

Analytical Methods

Enzyme Activity—The activity of all enzyme preparations was determined by measurement of leucyldiglycine hydrolysis. The stock substrate solution contained *dl*-leucyldiglycine (0.1 M), Na_2HPO_4 (0.05 M), NaCl (0.15 M), and $ZnCl_2$ (0.00015 M). To 1 cc. of this solution was added 0.5 cc. of suitably diluted enzyme. The pH of the mixture was approximately 7.05. One 0.5 cc. aliquot of the mixture was titrated immediately and another after 30 minutes incubation at 40.0°. The titrations were made by the acetone-HCl method of Linderstrøm-Lang (5). M/30 HCl being used, a titration increase of 0.5 cc. corresponded to complete hydrolysis of one linkage of the *l* component of the peptide. Duplicate determinations almost always agreed within less than 0.01 cc. The peptidase unit employed, which is identical with the unit used in previous papers, may be defined as the amount of enzyme which will bring about 50 per cent hydrolysis of 0.02 mole of *l*-peptide in 30 minutes. In the determination as described a

titration difference of 0.25 cc. corresponds to 0.0025 enzyme unit. Since the degree of hydrolysis is not strictly proportional to the quantity of enzyme present, an empirical curve must be constructed.

Nitrogen—The nitrogen content of enzyme preparations was determined by a colorimetric micromethod. A sample containing 10 to 40 γ of N was pipetted into an 18×150 mm. Pyrex test-tube, 1 cc. of 2 N H_2SO_4 containing 0.2 gm. per liter of $CuSeO_3$ was added, and the tube was covered by a loose glass cap. The contents of the tube were then digested overnight in a digestion rack kept in a 105° oven. The rack was provided with an electric heat source such that, after evaporation of the water, the H_2SO_4 condensed within 5 cm. of the bottom of the tube. To the tube, after digestion, were added in order 2 cc. of water, 2 cc. of color reagent, and 3 cc. of 2 N NaOH. The color reagent contained per liter 4 gm. of KI, 4 gm. of HgI_2, and 1.75 gm. of gum ghatti. After standing 15 minutes, the tube was placed in a photoelectric colorimeter and a reading taken. A 490 mμ filter was used. Blanks and nitrogen standards were always run with each series of samples. Only matched groups of tubes were used. The extinction coefficient was found to be proportional to the quantity of nitrogen for samples containing less than 45 γ of nitrogen. Known samples could be recovered with an error of 3 per cent or less.

Specific Activity—The specific activity of enzyme preparations was expressed as the number of enzyme units present per gm. of nitrogen in the preparation. The most active preparations thus far obtained have had specific activities in the neighborhood of 30,000. 1 gm. of such a preparation will hydrolyze 1.4 moles of *l*-leucyldiglycine per minute at 40°.

Hydrolysis Conditions—In all the hydrolysis experiments reported in this paper, the substrate concentration was M/30 with respect to available substrate; that is, M/15 in the case of *dl* mixtures. Since the acidic dissociation constant of the di- and tripeptides used is approximately 10^{-8}, in the neighborhood of pH 8 the substrates themselves are efficient buffers, and no further buffer need be added. The incubation temperature in all cases was 40° and the pH was 8.0 unless otherwise stated. All hydrolysis figures are expressed as per cent hydrolysis of one linkage of one optical component.

Preparation of Peptidase Protein

Autolysis—The yeast used was brewers' bottom yeast from a local brewery. Many types of yeast were investigated, and were found to vary greatly in their autolysis behavior. The one selected gave the best yield of enzyme. It was found that the yield was greatly improved if the pressed yeast (75 per cent moisture) was kept frozen in a cold room ($-6°$ to $-10°$) for a few months before being autolyzed. In the procedure finally adopted, the frozen pressed yeast was treated with an equal weight of water, covered with toluene, and allowed to autolyze, with occasional shaking, at room temperature ($22-25°$). The suspension always maintained itself at pH 6.1 without adjustment. Samples were removed at intervals for peptidase assay, and when there was no further increase in activity (3 to 5 days), the mixture was filtered with suction after the addition of Hyflo super-cel (a diatomaceous filter aid sold by Johns-Manville). The filter cake was washed with water and the washings added to the filtrate.

Acetone Precipitation—The procedure for isolation of the enzyme protein from the crude autolysate consists of a series of fractional acetone precipitations. The number of precipitations necessary, and the optimum pH and acetone concentration for each step, vary somewhat with each preparation. The precipitations are carried out as rapidly as possible, since the enzyme is not stable at high acetone concentrations. The general procedure is illustrated by the example of Table I. Often further fractional acetone precipitation is necessary to produce a preparation of high activity. During the latter stages of purification, a salt of a divalent metal is added to facilitate flocculation of the protein precipitate.

Various procedures involving adsorption, ammonium sulfate fractionation, acid precipitation, and other fractionation methods have not in general produced preparations any more active than can be obtained by repeated fractional acetone precipitation. Ammonium sulfate fractionation of fairly pure material, however, often is advantageous. The most active preparations thus far obtained have been prepared in this manner.

Fractions ranging in specific activity from 15,000 to 30,000 have been found to behave as homogeneous substances when tested in the Svedberg ultracentrifuge or the Tiselius electrophoresis ap-

paratus. When preparations, originally very active, which have decreased in activity during long storage have been fractionated, it has never been found possible to separate active from inactive material. All present data indicate the presence in all preparations of varying proportions of inactivated enzyme or of some other protein inseparable from the enzyme protein.

TABLE I
Isolation of Peptidase Protein

Step No.	Treatment	Volume	Total enzyme	Specific activity
		cc.	units	
I	1 kilo pressed brewers' yeast, frozen 3 mos., + 1 liter H_2O, autolyzed 4 days at pH 6.1; filtered; cell residue discarded; solution analyzed	2020	550	25
II	Solution adjusted to pH 5.7, acetone added to 28 volumes %; centrifuged; supernatant discarded; ppt. taken up in water; centrifuged; ppt. washed with water, washings added to supernatant; combined solution analyzed	246	440	1,060
III	Solution adjusted to pH 5.8, acetone added to 30 volumes %; centrifuged; ppt. dissolved in water, solution clarified in centrifuge before analysis	16.7	410	16,700
IV	Solution treated with $MgCl_2$ to 0.001 M, adjusted to pH 5.6, treated with acetone to 13.5 volumes %; ppt. centrifuged off, discarded; acetone added to 18 volumes %; ppt. centrifuged off, dissolved in water, analyzed	5.9	163	20,800
V	Residual 18% acetone solution from Step IV made to 26 volumes % acetone; ppt. centrifuged off, dissolved in water, analyzed	5.9	179	23,000

Properties of Peptidase Protein

Sedimentation, Diffusion, and Mobility[1]—A 0.2 per cent solution of the enzyme protein (specific activity 15,000) in 0.05 M (pH

7.05) phosphate buffer containing 0.1 M NaCl was sedimented in the Svedberg velocity ultracentrifuge. The sedimentation diagrams, obtained by the scale method, indicated that the material was homogeneous. The sedimentation constant at 20° was found to be 21.3×10^{-13} cm. sec.$^{-1}$ dyne^{-1}. A diffusion experiment performed at the same protein and buffer concentration showed the diffusion constant at 20° to be 3.09×10^{-7} cm.^{2} sec.$^{-1}$. On the basis of these two values, a provisional value of 670,000 for the molecular weight is obtained. Whether the enzyme exists at physiological concentrations as molecules of this size has not been determined.

As has been mentioned, samples of peptidase which do not possess maximal activity behave as homogeneous proteins. In order to obtain further information on this seeming anomaly, two preparations, one having a specific activity of 30,000 and the other a specific activity of 16,000, were compared on the Tiselius apparatus. The protein concentration in both cases was 0.2 per cent. A phosphate–NaCl buffer (pH 6.95 and ionic strength 0.07) was used. Both preparations were found to migrate as homogeneous substances. The more active sample had a mobility of 6.1×10^{-5} cm.^{2} sec.$^{-1}$, while the mobility of the less active preparation was 7.4×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

Since preparations which undoubtedly contain a large percentage of inactive material are homogeneous with regard to both sedimentation and electrophoretic migration, it seems likely that the inactive material present is a constituent part of the macromolecule. If this were true, the presence of the inactive material might affect the mobility, but not the homogeneity of the sample.

Herriott, Desreux, and Northrop (6) have reported that pepsin preparations containing inactive protein are electrophoretically homogeneous, although their solubility behavior indicates inhomogeneity.

Other Properties—The enzyme is soluble in water at pH values above 5.2 to 5.3. It may be precipitated from aqueous solution by acidification to pH 4.5 to 4.8. At pH values below 4.0 it is soluble, but is very rapidly inactivated. In dilute salt solutions it is soluble at all pH values. It is soluble in 0.6 saturated ammonium sulfate solution, but relatively insoluble in 0.8 saturated ammonium sulfate solution.

Neutral aqueous solutions of the enzyme containing a few gm. of protein per liter are stable for weeks at 2°. In very dilute aqueous solution, the enzyme is rather unstable. Its stability is a function of pH. In Fig. 1 is shown the effect of pH on the stability of the enzyme in a solution containing about 2.4 mg. of protein per liter. The enzyme is unstable in neutral solution, but is relatively stable at higher pH values. It has been found that in the presence of 0.01 M NaCl enzyme solutions are perfectly stable for 2 hours at pH 7, but not at pH 5.6. Because of this instability of the protein in dilute aqueous solution, enzyme solu-
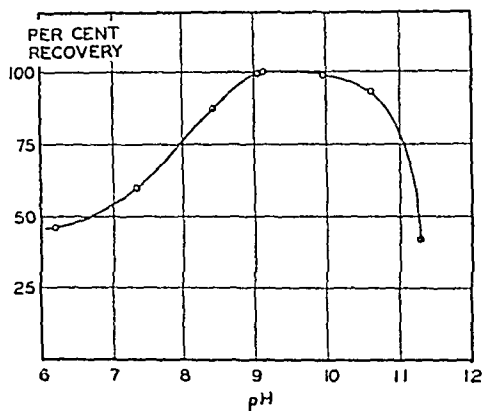
Fig. 1. Stability of a dilute solution of peptidase protein held 3 hours at 22° in 0.001 M borate plus 0.001 M phosphate. Preparation 878, specific activity 17,200; 0.3 γ of enzyme N per cc.

tions were always diluted for assay sampling with 0.001 M borate buffer of pH 9.2.

The chemical composition of the enzyme protein has not yet been studied. Orientation experiments have shown it to contain about 13.5 per cent N and 0.3 per cent P. Acid hydrolysis liberates approximately 5 per cent of sugar as determined by copper reduction and orcinol color methods.

Enzymatic Activity of Protein

Specificity—Table II summarizes data on the hydrolysis of various substrates by the enzyme protein. Tripeptides are hydrolyzed more rapidly than corresponding dipeptides. *dl*-Leucyl-methylamine (leucyldecarboxyglycine) is split, showing that a

carboxyl group is not essential for hydrolysis. The slow hydrolysis of *dl*-prolyldiglycine indicates that an intact amino group is not necessary. *dl*-N-Methylleucyldiglycine, however, is not split. The data of Table III show the enzyme to have a high degree of optical specificity. *l*-Leucyldiglycine is hydrolyzed 5000 times as rapidly as the unnatural *d*-leucyldiglycine. If the

TABLE II

Hydrolysis of Various Substrates by Yeast Polypeptidase

Preparation S40, specific activity 16,300; 0.1 M NaCl present.

Substrate	Enzyme N	Incubation time	Hydrolysis
	γ per cc.	hrs.	per cent
dl-Leucyldiglycine	0.091	0.5	55
dl-Alanyldiglycine.....	0.253	0.5	53
Triglycine.....	0.253	8	56
dl-Leucylglycine........ ..	0.253	2	64
dl-Alanylglycine............ .. .	0.253	8	39
Diglycine.....................	25.3	2	30
dl-Leucylmethylamine...	0.253	8	36
dl-Prolyldiglycine.......	25.3	2	73
dl-N-Methylleucyldiglycine..	25.3	8	0

TABLE III

Optical Specificity of Yeast Polypeptidase

Preparation 913, specific activity 21,000; 0.1 M NaCl and 0.0001 M ZnSO₄ present.

Substrate	Enzyme N	Incubation time	Hydrolysis
	γ per cc.	hrs.	per cent
l-Leucyldiglycine	0.107	0.5	80
d-Leucyldiglycine	13.4	0.5	1
		5	20
		22	76

d-leucyldiglycine used had been contaminated with any of the *l* form, a greater degree of hydrolysis would have occurred during the first half hour. It should also be mentioned that the enzyme hydrolyzes the peptide linkage adjacent to the amino group. When leucyldiglycine is hydrolyzed by a large amount of enzyme, hydrolysis proceeds rapidly until one linkage has been split.

Further hydrolysis is relatively slow.   If leucylglycine instead of diglycine were a primary hydrolysis product, further hydrolysis would be much more rapid.   When the experiment is performed, the rate of secondary hydrolysis corresponds to the rate at which diglycine is split by the enzyme and not to the more rapid rate at which leucylglycine is split.   Since leucylglycine is split 300 times as fast as diglycine, the difference is accurately observable.

Activation—The activity of the peptidase is greatly affected by the presence of chloride ions and zinc ions.   The data of Table IV show that its activity in the presence of both zinc and chloride is much greater than the activity in the presence of either alone.   Such behavior indicates that both ions are in some way necessary

TABLE IV

Effect of NaCl and ZnSO₄ on Enzyme Activity

Preparation 913, specific activity 21,000.

Substrate	Added NaCl	Added ZnSO₄	Enzyme N	Incubation time	Hydrolysis
	M	M	γ per cc.	hrs.	per cent
dl-Alanyldiglycine	0.0	0.0	0.053	1.0	2
	0.1	0.0	0.053	1.0	12
	0.0	0.0001	0.053	1.0	8
	0.1	0.0001	0.053	1.0	58
dl-Leucyldiglycine	0.0	0.0	0.067	0.5	5
	0.1	0.0	0.067	0.5	14
	0.0	0.0001	0.067	0.5	10
	0.1	0.0001	0.067	0.5	44

in the hydrolysis process.   The zinc concentration necessary for maximal activation is rather low.   The experiment summarized in Table V shows 10^{-4} M to be approximately optimal.   The optimal NaCl concentration is high, as may be seen from Table VI.   While 0.0001 M NaCl gives a readily detectable effect, greatest activity was obtained in the presence of 0.1 M NaCl.   At concentrations much greater than this, precipitation occurs during the acetone titration.

Salts of Al′′′, Ca′′, Cd′′, Co′′, Cu′′, Fe′′, Hg′′, Mg′′, Mn′′, Ni′′, Pb′′, and Zn′′ were tested for activating effect on alanyldiglycine hydrolysis in the presence of 0.1 M NaCl.   The metals were tested at 10^{-5} M, 10^{-4} M, and 10^{-3} M.   Only Zn′′ gave good activation.

Co″, however, gave at 10^{-4} M a definite and reproducible, though relatively small (60 per cent) activation.

The activation by sodium chloride is due to the chloride ion rather than the sodium ion; $MgCl_2$ is an effective activator, while

TABLE V

Effect of ZnSO₄ Concentration on Enzyme Activity

Preparation 913, specific activity 21,000; 0.067 γ of N per cc.; incubation time 30 minutes; substrate *dl*-leucyldiglycine.

Added NaCl	Added ZnSO₄	Hydrolysis
M	M	per cent
0.1	0	14
0.1	10^{-6}	16
0.1	10^{-5}	28
0.1	10^{-4}	46
0.1	10^{-3}	43
0.0	0	5
0.0	10^{-4}	10

TABLE VI

Effect of NaCl Concentration on Enzyme Activity

Preparation 959, specific activity 16,300; 0.091 γ of N per cc.; substrate *dl*-alanyldiglycine; pH 7.2; incubation time 30 minutes; 0.0001 M ZnSO₄ present.

Added NaCl	Hydrolysis
M	per cent
0	19
10^{-5}	20
10^{-4}	26
10^{-3}	44
10^{-2}	56
10^{-1}	65
10^{-2}	12*

* No ZnSO₄ added.

sodium sulfate and sodium acetate are not (Table VII). Moreover the sodium hydroxide necessary to adjust the peptide substrates to pH 8 brings the sodium ion concentration to approximately 0.033 M even in the absence of added activator. Yet the

addition of 0.001 M NaCl to such a substrate more than doubles the activity of the enzyme (Table VI).

Ordinary preparations of leucyldiglycine prepared by the method of Fischer (7) were found to be hydrolyzed as rapidly in the absence of added zinc as in its presence. Repeated recrystallization was necessary to obtain preparations which would give maximal zinc activation. It was found that the trace of ash present in ordinary preparations would, if added to recrystallized preparations, greatly promote hydrolysis. Since peptides prepared from the corresponding α-halogen acyl compounds usually contain traces of ammonium halide, recrystallization was often necessary to obtain maximal NaCl activation. It seems probable

TABLE VII

Effect of Anions on Enzyme Activity

Preparation 878, specific activity 17,200; 0.105 γ of N per cc.; substrate *dl*-leucyldiglycine.

Salt added	Concentration	Hydrolysis
	M	per cent
None	0	32
NaCl	0.01	60
Na$_2$SO$_4$	0.005	35
NaOOCCH$_3$	0.02	26
NaBr	0.01	58
KI	0.01	52
MgCl$_2$	0.005	56
NaNO$_3$	0.01	44

that the activity observed in the absence of added activators is due to the presence of traces of zinc and halogens in the peptide preparation and in the NaOH used for pH adjustment.

pH Optimum—In Fig. 2 pH-activity curves for the hydrolysis of leucyldiglycine and alanyldiglycine are given. It will be seen that leucyldiglycine is attacked most rapidly at pH 7.9, while alanyldiglycine is most rapidly split at pH 7.0. The leucyldiglycine substrate used did not contain added zinc, but addition of zinc salts had no effect at either pH 7 or 8. The enzyme is stable at pH 10, as may be seen from Fig. 1, but no enzymatic activity was evident at this pH. In the acid pH region, however, it is possible that instability of the enzyme may have affected the shape of the curves of Fig. 2. The leucyldiglycine substrate used

for routine peptidase assay has a pH of 7.05. This pH value was originally adopted because it is the optimal pH for leucyldiglycine hydrolysis by crude yeast autolysate.

Relation of Yeast Polypeptidase to Other Peptidases

The enzyme described above is not identical with the yeast aminopolypeptidase described by Grassmann and his associates. Their enzyme hydrolyzed leucyldiglycine 500 times as rapidly as
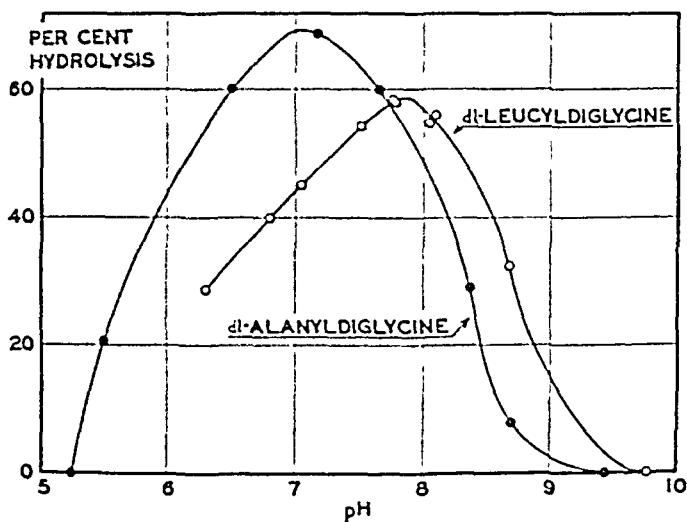
FIG. 2. pH-activity curves for peptide hydrolysis. dl-Leucyldiglycine, enzyme Preparation 840, specific activity 16,300; 0.091 γ of N per cc.; 0.1 M NaCl present; incubation time 30 minutes. dl-Alanyldiglycine, enzyme Preparation 913, specific activity 21,000; 0.08 γ of N per cc.; 0.1 M NaCl and 0.0001 M ZnSO₄ present; incubation time 60 minutes.

leucylglycine (4), while the present peptidase hydrolyzes the tripeptide only 8 times as rapidly as the dipeptide when substrates of the same composition as those used by Grassmann are employed. Moreover, Grassmann's enzyme splits triglycine 40 per cent as fast as leucyldiglycine (8), whereas the enzyme described in this paper splits triglycine only 3 per cent as rapidly as leucyldiglycine. Grassmann (9) has stated that yeast aminopolypeptidase acquires dipeptidase activity in the presence of chlorides. Such behavior seems to indicate the presence in his preparations of a chloride-activated peptidase.

In a previous publication from this laboratory (10) the resemblance of a previously studied mold polypeptidase (11) to yeast polypeptidase was shown. Both enzymes are activated by zinc, both enzymes split tripeptides rapidly and dipeptides slowly, and both enzymes split leucyl peptides much more rapidly than glycyl peptides.

Many molds possess a similar peptidase. Although the mold peptidases were not found to be activated by chloride ions, the many similarities seem to warrant a provisional conclusion that the zinc-activated yeast polypeptidase is one of a group of similar peptidases more or less widely distributed in nature.

SUMMARY

A protein having high polypeptidase activity has been isolated from brewers' yeast. The protein behaves as a homogeneous substance on electrophoresis and ultracentrifugal sedimentation. It has a molecular weight in the neighborhood of 700,000. It has not been obtained in a crystalline state. The polypeptidase splits tripeptides much more rapidly than dipeptides. It hydrolyzes the peptide linkage adjacent to the free amino group of the peptide. It does not require a carboxyl group. The presence of zinc ions and halide ions appears to be necessary for its activity.

A convenient micro-Kjeldahl nitrogen method is described.

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THE DETERMINATION OF SODIUM

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We have adopted the method of Butler and Tuthill (1) for determining sodium in plasma, using slight modifications that will be described below. According to our experience with this method, the plasma of healthy men contains about 140 milliequivalents per liter, most values lying between 138 and 142.

Hald (2), in Peters' laboratory, has described a modification of the Butler-Tuthill procedure that gives values ranging from 133 to 138 milliequivalents. We have looked with doubt on her low figures and she questions the validity of the higher values obtained by most other investigators (3 6). Some observations are reported here which seem to resolve the problem.

The techniques used differ somewhat from those originally described by Butler and Tuthill. In order to avoid ambiguities a detailed description will be given.

Delivery of Sample—Either a Normax volumetric pipette, a Van Slyke stop-cock pipette, or an automatic syringe pipette may be used. All are sufficiently exact, the last being most precise. The syringe pipette may be used by rinsing it with the material to be analyzed and then delivering the sample, or it may be rinsed with distilled water between deliveries, the rinsings being added to the sample. The former procedure is somewhat wasteful of material, since three rinsings, one each with 0.2 ml. of sample, are necessary even when successive samples are known to have approximately the same composition. The latter procedure gives a 2 ml. sample, since five rinsings are necessary. In most cases W. V. C. used the automatic syringe and D. B. D. the Van Slyke pipette.

Container Used for Ashing—Either a platinum crucible of 30

ml. capacity or one of 5 ml. capacity or a cone-tipped silica tube of 10 ml. capacity may be used (7). D. B. D. used the first and W. V. C. the others.

Preparation for Ashing—In the case of plasma 1 drop of concentrated H_2SO_4 or 0.5 ml. of 10 per cent H_2SO_4 was added to the plasma sample. W. V. C. used the same quantity with the inorganic unknowns, while to them D. B. D. added only 2 drops of 10 per cent H_2SO_4. In the round bottom platinum dish the larger quantity of H_2SO_4 tends to creep during the early stage of ashing in the absence of organic material. The sample thus treated with H_2SO_4 is dried in an oven at 110° for 1 or 2 hours if a platinum dish is used, or for 12 hours if a silica tube is used.

Ashing—The dishes are placed in a cold furnace and the rheostat set so a maximal temperature of 500° will be reached in a few hours. Ashing is completed overnight.

Solution and Transfer of Ash—About 15 ml. of the freshly filtered uranyl reagent are poured into the Jena crucible, as prescribed by Butler and Tuthill. The ash is dissolved in 0.5 ml. of N HCl and the dish rotated gently until solution is complete. The uranyl reagent is mechanically stirred, with a glass rod about 3 mm. in diameter, while the sample is being transferred with a capillary pipette. Two rinses are made with about 0.3 ml. of 0.1 N HCl and finally 2 ml. of the uranyl reagent are used to rinse dish and pipette. After the stirring has continued for 5 minutes, the rod is removed and rinsed with reagent, and the dish is covered and is set aside for 1 hour before filtering. The subsequent steps are carried out as described by Butler and Tuthill.

Removal of Phosphate—In Hald's method emphasis is placed on the removal of phosphate, and she accomplishes this by a ferric phosphate procedure.[1] We have employed her procedure in some instances and in others have added 1 gm. of powdered $Ca(OH)_2$

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[1] Although Hald says that known solutions containing sodium and KH_2PO_4 can be dry-ashed without loss of phosphorus, this is not true of plasma to which sulfuric acid has been added. Sulfuric acid boils at about 330°, while phosphoric acid begins to volatilize appreciably at from 260–300°. On samples of plasma ashed by our procedure we find phosphorus concentrations ranging from 1 to 7 mg. per cent. Evidently from one-half to nearly all the "total" phosphorus may be lost from plasma dry-ashed in the presence of sulfuric acid.

to 10 ml. of dissolved ash, as recommended for urine by Butler and Tuthill. *This is shaken for 1 hour, centrifuged, and an aliquot taken for analysis.*

Testing Hald's Technique—Porcelain dishes were used both with and without phosphate removal. Hald's procedure for removing phosphate calls for the addition of 1 drop of 1.29 per cent ferric sulfate, the addition of 2 to 4 cc. of sulfuric acid, 4 N, drying, and ashing. The ash is taken up in hot distilled water, made up to volume, and centrifuged. An aliquot is evaporated

TABLE I
Sodium Determinations in Standard Solutions

Solution	Present		Na found	
	P	Na	By W. V. C.	By D. B. D.
	mg. per l.	*m.eq. per l.*	*m.eq. per l.*	*m.eq. per l.*
A	125.5	131.6	133.4	131.8
			133.4	133.5
B	115.9	142.4	141.6	141.1
			141.4	141.9
C	36.2	137.3	136.7	136.8
			136 5	136.4
D	62.2	127 9	126 9	127.7
			127.3	127.5
E	147.0	138.2	137.6	137.4
			137.6	138.3
Mean error.....			−0.7	−0.8

and transferred to the uranyl reagent. We have also employed this technique in quartz tubes.

Analyses were made independently by the authors of solutions precisely prepared for us by Professor S. G. Simpson of the Department of Chemistry, Massachusetts Institute of Technology. These contained Na, K, Ca, Mg, and HPO_4 within or near the physiological range and, in addition, Cl and SO_4. The results of our analyses, made without removing phosphate, are compared in Table I with data subsequently furnished by Professor Simpson. With the exception of one poor value obtained by D. B. D., the recovery ranges from 99.1 to 100.1 per cent.

One of the authors (W. V. C.) proceeded to try other containers

for ashing and variations in the technique. The results are shown in Table II. They suggest that any sort of ashing is apt to produce a loss. In our hands Hald's procedure, coupled with the use of porcelain dishes, resulted in an average loss of 2.9 milliequivalents in these inorganic solutions in contrast to a loss of

TABLE II

Deviations, in Milliequivalents, from True Value with Various Procedures

Procedure	Solution				
	A	B	C	D	E
Unashed; Butler-Tuthill technique	−0.1	−0.1	+0.2	−1.0	−0.1
Ashed in SiO₂; Butler-Tuthill technique..	−1.2	−0.9	−0.7	−0.8	−0.6
Ashed in SiO₂; P removed with Ca(OH)₂	−1.3	−0.1	−0.3	−0.4	−0.4
Ashed in SiO₂; P removed by Stadie-Ross (8) technique	−1.1	−1.4	−0.4	−0.1	−1.2
Ashed in SiO₂; P removed and ash dissolved by Hald's technique .	−3.4	−1.8	−0.6	−1.1	−3.9
Ashed in porcelain; P removed by Hald's technique	−3.5	−3.7	−0.8	−2.4	−4.1

TABLE III

Determination of Sodium in Human Plasma under Various Conditions
The results are expressed in milliequivalents per liter.

	P not removed			P removed	
Container	Platinum dish	SiO₂ tube	Porcelain dish	Platinum dish or SiO₂ tube Ca(OH)₂ used	Porcelain dish
Method	Ours	Ours	Ours		Hald's
No. of analyses .	10	10	10	4	9
Maximum ...	140.9	140.7	139.4	140.6	136.8
Minimum...	139.5	139.5	136 0	140.3	135.5
Average ..	140.2	140.2	138.3	140.5	136.1

about 0.8 milliequivalent found by the authors using platinum and the technique described herein.

The implication of these findings is that some sodium is trapped in the neutral ferric phosphate precipitate. To test this the collected precipitates from ten determinations by Hald's method,

in which porcelain dishes were employed, were washed, dissolved, and the phosphate reprecipitated by rendering the solution alkaline with ammonia. The supernatant fluid contained an amount of sodium equivalent to 2 milliequivalents per liter in the original solution. This amount is of the order of magnitude of the deficiency found by the Hald technique as we applied it to these inorganic solutions.

An application of these findings was next made to plasma, as shown in Table III. The average recovery of sodium from ten consecutive samples of a normal human plasma was 140.2 milliequivalents when ashing was carried out in platinum or silica without removing phosphate. In a second series, ashing was carried out in porcelain and phosphate was removed by Hald's technique. The average concentration was 136.1 milliequivalents, a difference of −4.1. The phosphate residues were combined, washed, dissolved, and reprecipitated. The sodium recovered corresponded to 3.5 milliequivalents. It is certain, therefore, that in our hands the Hald technique is defective in that sodium is left undissolved when the ash is leached with distilled water.

SUMMARY

Hald's technique for sodium when applied to inorganic solutions containing other electrolytes gives results that may be too low by 4 milliequivalents per liter. Her method of leaching the ash with distilled water evidently leaves some sodium undissolved. With the Butler-Tuthill technique slightly modified, recoveries averaged 99.5 per cent from these solutions. Contrary to Hald's report, no values were too high when phosphate was not removed.

The applications of these findings to plasma revealed a slight loss of sodium when porcelain was used for ashing and a considerable loss when ferric sulfate was added before ashing and water was used for leaching the ash. It is concluded that the latter procedure, *viz.* that of Hald, gives results that are too low by about 3 or 4 milliequivalents.

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STUDIES ON BIOLOGICAL OXIDATIONS

XIV. OXIDATIONS BY MICROORGANISMS WHICH DO NOT FERMENT GLUCOSE*

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There are two methods of approach to the study of the mechanisms of biological oxidations, (1) the isolation of the components of the enzyme system and (2) work with living cells in which the oxidations proceed very simply, without intermediate reactions. The splendid achievements of Warburg and his coworkers on the isolation of some enzyme components have had the usual reaction among scientists interested in the study of biological oxidations— a diligent application of Warburg's methods and consequent neglect of the second mode of approach. Although the first mode of approach brings forth more satisfactory results, it cannot be denied that the second is more likely to reveal varied mechanisms in the oxidation of a substrate. Bacterial cells are excellent material for this second method, because they may be grown in a very short time, and, in general, the oxidation reactions are not masked by regulating mechanisms such as exist in animal cells.

Two problems were the concern of this investigation. (1) Are bacteria which usually do not ferment glucose also unable to oxidize it, and if so, why? (2) Are the oxidations in these bacteria all performed by the same mechanism? An answer to the first problem was considered essential for the proper understanding of the metabolism of glucose; an answer to the second was considered valuable as an indication of the unity or plurality of enzymes oxidizing *one particular substrate.*

The experiments reported in this paper show that glucose is oxidized by a number of bacteria without previous fermentation

(anaerobic scission), and that in cases in which the glucose molecule is not oxidized it becomes oxidizable as soon as the molecule is phosphorylated, an indication that phosphorylation is the essential primary process for the metabolism of glucose. The isolation of the components of two oxidation enzymes (hexose monophosphate oxidase from yeast, and d-amino acid oxidase from kidney) gave an opportunity to test whether oxidation-enzyme systems possess the same components in all living cells. Strong arguments against such an assumption arise from the observations of Barron and Jacobs (1) on the oxidation of lactic acid by hemolytic streptococcus. Those offered here, based on the inhibition by HCN of the oxidation of hexose monophosphate and d-amino acids by all the bacteria studied, are further evidence that the oxidation components of individual enzyme systems are not uniform but may vary from one type of cell to another.

EXPERIMENTAL

The bacteria were grown in flat medicine bottles in a medium containing 1 per cent of proteose-peptone, 0.3 per cent of meat extract, 0.5 per cent of NaCl, and 3 per cent of agar, all dissolved in tap water. After 24 hours, 15 to 20 cc. of sterile 0.9 per cent NaCl solution were added and the bacteria were removed from the surface of the medium. They were resuspended twice in the saline solution and finally suspended in a salt solution prepared according to Krebs and Henseleit (2). To 90 cc. of this suspension were added 10 cc. of 0.2 M phosphate buffer, pH 7.06. The oxygen consumption was measured with the usual Barcroft-Warburg manometers at 38° with air as the gas phase. The anaerobic breakdown was determined by measuring the CO_2 production of bacteria suspended in the same salt mixture with bicarbonate buffer and N_2-CO_2 (95:5) as the gas phase, the gas being previously passed through a 1 meter long Pyrex glass tube filled with Cu pellets and heated to 500°. Calcium hexose diphosphate was obtained from The British Drug Houses, Ltd.; calcium hexose monophosphate was kindly provided by Dr. C. V. Smythe, and adenine triphosphate by Dr. Y. Subbarow, to both of whom we express our thanks. Recrystallized lithium lactate, pyruvate, and α-ketoglutarate were prepared in the laboratory. All experiments were repeated at different intervals of time.

The results given are the averages of three series of triplicate experiments in which the individual variations never exceeded 5 per cent. These results are stated in terms of mg. of dry weight as a matter of convenience only. We are well aware that any method of giving quantitative results for bacteria fails to distinguish cells with no metabolic activity from those of varied activity. Furthermore, we recognize the possibility of the presence of small quantities of other solid matter, such as bacterial debris and particles of agar. Single strains of the following bacteria were studied, *Pseudomonas aeruginosa*, *Phytomonas campestris*, *Micrococcus piltonensis*, *Micrococcus freudenreichii*, *Sarcina lutea*, and *Gaffkya tetragena*. Three strains of *Alcaligenes faecalis* were studied, (1) from the collection of one of us (T. E. F.) isolated in 1930, (2) from the Department of Bacteriology of the University of Chicago, and (3) from the Department of Bacteriology of Northwestern University. Each of these had been kept many years on "plain" agar medium, which contains no added fermentable sugar. Suspensions of pneumococcus, Type I, were prepared by Miss Gladys Peterson in the laboratory of Dr. O. H. Robertson.

Oxidation and Fermentation of Glucose—Glucose is oxidized by most bacteria. Whether it is directly oxidized or is previously split into more easily oxidizable components is not yet known. As the work of Embden *et al.* (3), Parnas (4), Meyerhof (5), Warburg and Christian (6), and Cori *et al.* (7) unraveled the series of reactions that takes place in glycolysis and alcoholic fermentation, a number of investigators (Virtanen and Tikka (8), Neuberg and Kobel (9), Werkman *et al.* (10–12), and Endo (13)) demonstrated that different species of bacteria are able to phosphorylate glucose. These investigations have led to the belief that the metabolism of glucose by bacteria follows the same cycle of reactions as in muscle extracts, and to the assumption that bacteria unable to split glucose anaerobically are also unable to oxidize it (Hansen (14)). It must be stated, however, that there have been protests against this unitarian conception of glucose metabolism; witness, Dickens' suggestion (15) that glucose might be oxidized by yeast soon after phosphorylation, and Lundsgaard's (16) and Shorr, Barker, and Malani's (17) reports that respiration proceeds in yeast and mammalian tissues even after fermentation has been inhibited by iodoacetic acid.

It is generally admitted (see Bergey (18)) that *Sarcina lutea, Micrococcus piltonensis, Phytomonas campestris,* and *Alcaligenes faccalis* do not ferment glucose in culture media, as indicated by acid formation. *Gaffkya tetragena* and *Micrococcus freudenreichii,* it is stated, produce acid from dextrose. Sandiford (19) has found that *Pseudomonas aeruginosa* (which was previously classified among the non-fermenting bacteria) produces acid from glucose, although "as judged by the colour change, the amount of acid produced was small." On the other hand, Kendall, Friedemann, and Ishikawa (20) found that suspensions of *Pseudomonas aeruginosa* incubated with glucose at 45° did not utilize this substance appreciably.

In experiments of 2 hours duration (Table I) the amount of CO_2 produced by these bacteria in the absence of oxygen, with the exception of *G. tetragena*, was the same whether glucose was present or not. Data from Barron and Miller's studies (21) on the metabolism of gonococci have been added to show that under identical conditions gonococci produce large amounts of acid, as indicated by the CO_2 output. A strain of *G. tetragena*, freshly isolated from pus from an ear infection, produced with glucose 19 c.mm. of CO_2, more than 3 times as much as the old strain from our collection (5.1 c.mm.). In these experiments the lactic acid formation was determined by both the Warburg manometric method and the method of Friedemann and Graeser (22). The agreement was satisfactory. However, the yield of acid from the recently isolated strain was far less than was obtained from gonococci and pneumococci. The greater anaerobic metabolism of the freshly isolated strain may be an indication that in some bacteria the specific proteins for the fermentation of glucose are present in greater concentration while the bacteria are in the animal host and that they are gradually lost when grown for a long time in culture media without glucose.

In the presence of oxygen all these bacteria oxidized glucose with the exception of *M. freudenreichii* and *A. faecalis*, Strain 2. The oxidation of glucose by *Ps. aeruginosa, G. tetragena, S. lutea, Ph. campestris,* and *A. faecalis*, Strain 1, was accompanied by the formation of CO_2. The respiratory quotient due to glucose oxidation, determined by subtracting the O_2 uptake and the CO_2 output of the bacteria in the absence of glucose, was about 1. *M.*

Table I

Metabolism of Glucose

The fermentation results are given as CO_2 output in bicarbonate buffer, pH 7.4, with N_2-CO_2 as the gas phase; those for oxidation as O_2 uptake in phosphate buffer, pH 7.06, with air as the gas phase. The figures give c mm. per hour per mg. of dry weight. Temperature, 38°; amount of glucose, 0 03 mм.

Bacteria	CO_2 output		O_2 uptake		Inhibition of oxidation	
	With-out glucose	With glucose	With-out glucose	With glucose	NaF	CH₂-ICOOH
	c.mm.	c.mm.	c mm.	c mm.	per cent	per cent
Ps. aeruginosa............	17 7	16.1	9 6	151 1	None	41.6
Ph. campestris..............	13 5	13 0	10 9	75 5	19 4	43.1
G. tetragena (old culture)	12 0	17 1	11 7	35.9	26 0	72 0
" " (freshly isolated).	5 0	24 4	6 3	76 6		
S. lutea..	12 7	12 7	8 4	23 2	24 8	46.1
M. piltonensis	25 5	24 6	0	10 0	42 0	None
A. faecalis, Strain 1 . .			7 5	24 3	None	
" " " 2 ..			10 4	10 4		
" " " 3....			9 0	20 0	None	
M. freudenreichii			10 2	9 7		
Gonococci	3 0	60 2	0	294 5	90 0	95 0
Pneumococci, Type I	2 0	52 1	0	72 0	91 4	96 0

Table II

Glucose Oxidation by Bacteria Which Do Not Ferment Glucose

Temperature, 38°; pH, 7.4, buffer, Ringer-phosphate, amount of glucose, 0.01 mм; duration of incubation, 4 hours.

Bacteria	Control		Glucose		R Q due to glucose oxidation
	O_2 uptake	CO_2 output	O_2 uptake	CO_2 output	
	c mm.	c mm.	c mm.	c.mm.	
Ps. aeruginosa .	182.8	184.3	1245 0	1264 4	1 02
S. lutea....... .	53 2	47 5	195 5	201 1	1 08
Ph. campestris.	260 5	221 5	1165 8	1157.2	1 03
A. faecalis, Strain 1.....	210.5	179 1	448 3	447.8	1.13
M. piltonensis..........	29 4	33 6	60 0	18 0	
G. tetragena.............	52.5	47.5	174.0	181 4	1.10

piltonensis oxidized glucose without CO_2 formation (Table II). *Ps. aeruginosa* seems to oxidize glucose to completion, for in one

experiment of 5 hours duration there was an O_2 uptake, due to the oxidation of 0.01 mM of glucose, of 1233 c.mm. (The total oxidation to CO_2 and H_2O of this amount of glucose requires 1344 c.mm. of O_2 uptake.) It should be recalled that Warburg and Christian (23) found that hexose monophosphate could be oxidized in the presence of yeast proteins, triphosphopyridine nucleotide, and alloxazine up to an uptake of 3 moles of O_2 per mole of hexose monophosphate, the R.Q. being 1. The conclusion may be drawn that there are certain bacteria in which the processes of fermentation and oxidation of glucose are sharply separated, since bacteria unable to ferment glucose (S. lutea, Ph. campestris, M. piltonensis, Ps. aeruginosa) possess the power to oxidize it.

Effects of NaF and CH₂ICOOH—NaF inhibits the fermentation of glucose (glycolysis) in muscle extract by stopping the transformation of phosphoglyceric acid into phosphopyruvic acid (Lohmann and Meyerhof (24)). If the oxidation of glucose does not proceed without previous conversion into pyruvic or lactic acid, NaF will act as an inhibitor. It happens thus in the oxidation of glucose by gonococci (Barron and Miller (21)), by hemolytic streptococci (Barron and Jacobs (1)), and by pneumococci (Table I), where 0.02 M NaF inhibits the oxidation of glucose almost completely. In the present experiments (Table I), NaF (0.02 M) had no effect on the oxidation of glucose by Ps. aeruginosa and A. faecalis, Strain 1; there was an inhibition of 19 per cent on the oxidation by Ph. campestris, of 25 and 26 per cent respectively on the oxidation by S. lutea and G. tetragena, and of 42 per cent in M. piltonensis.

Iodoacetic acid in small concentrations (0.001 M) is also an inhibitor of glycolysis. It inhibits the oxidation of triosephosphoric acid to phosphoglyceric acid in muscle extract (Embden and Deuticke (25)). CH₂ICOOH (0.005 M) had no influence on the rate of oxidation of glucose by M. piltonensis, and inhibited by less than 50 per cent the rate of oxidation by S. lutea, Ph. campestris, and Ps. aeruginosa (Table I). The inhibiting effect is complete in sugar-fermenting bacteria (gonococci, hemolytic streptococci, pneumococci). There is thus a marked difference between bacteria which readily ferment glucose and those which do not ferment it appreciably. In the first group the oxidation of glucose is largely inhibited by NaF and CH₂ICOOH; in the second group the inhibition does not take place, or is only partial.

Oxidation of Hexose Phosphates, Lactate, and Pyruvate—Since the oxidation of glucose by the non-glucose-fermenting bacteria takes place before its anaerobic breakdown into C_3 compounds and is not merely an oxidation of glucose to gluconic acid, it may be postulated that glucose is oxidized only after its phosphorylation and that bacteria which are unable to oxidize glucose lack this power because they do not possess, or have lost, the power of phosphorylation. This postulate would imply that phosphorylated hexoses can be oxidized by these bacteria. As a matter of fact, *A. faecalis*, Strain 2, and *M. freudenreichii*, which oxidized no glucose, did oxidize both hexose phosphates readily. Further-

TABLE III

Oxidation of Hexose Phosphates and Lactate

Temperature, 38°; pH, 7.06; amount of substrates, 0.01 mM. The figures give c.mm. of O_2 uptake per hour per mg. of dry weight due to oxidation of the substrate (O_2 uptake without the substrate subtracted).

Bacteria	O_2 uptake		
	Hexose diphosphate	Hexose monophosphate	Lactate
Ps. aeruginosa..	86 4	122.3	219.0
Ph. campestris...	• 4 0	28.4	2.3
G. tetragena (old culture).........	106.0	142.5	84.2
S. lutea....	83.7	90.5	88.0
M. piltonensis	47.8	30.0	115.0
A. faecalis, Strain 1	8.2	42 5	77.0
" " " 2 .	12.5	57.0	123.0
" " " 3 .	1.5	25.7	98.0
M. freudenreichii.......	12.0	35.0	46.4

more, *G. tetragena*, *S. lutea*, *M. piltonensis*, and *A. faecalis*, Strains 1 and 3, which oxidized glucose at a slow rate, oxidized both hexose diphosphate and monophosphate at a higher rate (Table III).

The enzyme components for the oxidation of hexose monophosphate have been isolated by Warburg and Christian (26) (protein, triphosphopyridine nucleotide, alloxazine). If these enzyme components alone were responsible for the oxidation of hexose monophosphate by these bacteria, the rate of oxidation would be unaffected by HCN. *M. piltonensis* and *A. faecalis*, Strain 2, oxidized hexose monophosphate to phosphohexonic acid with an uptake of 1 atom of oxygen per mole of substrate, but the oxida-

tion was inhibited by HCN (Fig. 1). Quite recently Haas, Horecker, and Hogness (27) in a preliminary note have reported the presence in yeast of a flavin-mononucleotide protein which acts as an electron mediator between the sluggish systems triphosphopyridine nucleotide and cytochrome *c*. A similar enzyme component may act on the oxidation of hexose monophosphate by HCN-sensitive bacteria.

Since the enzyme concerned with the phosphorylation of glucose (phosphorylase) is composed of a protein and adenine tri-
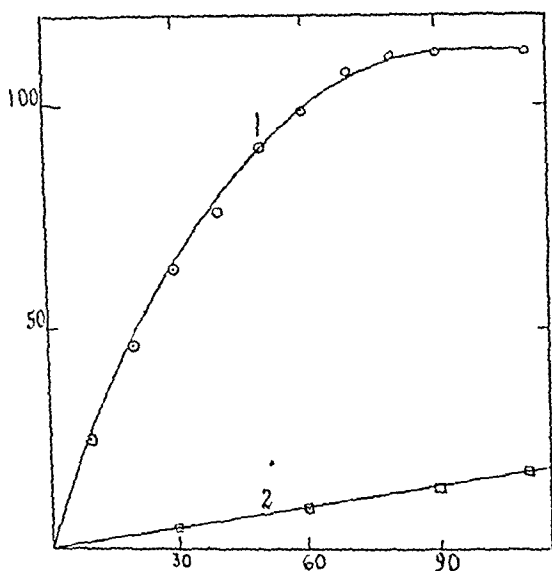
Fig. 1. Effect of HCN on the oxidation of hexose monophosphate by *Micrococcus piltonensis*. The abscissa represents time in minutes; the ordinate, O_2 uptake in c.mm. Curve 1, control; Curve 2, HCN (0.005 M). Amount of hexose monophosphate, 0.01 mM; pH, 7.06; temperature, 38°.

phosphate, an attempt was made to induce the oxidation of glucose by *M. freudenreichii* and *A. faecalis*, Strain 2, by adding adenine triphosphate to the bacterial suspension. Under these conditions, there was possibly slight oxidation of glucose by *M. freudenreichii*, but none at all by *A. faecalis*, although adenine triphosphate alone was oxidized by both bacteria, quite vigorously by *A. faecalis*. Lack of glucose oxidation may be due to lack of the protein concerned with phosphorylation (Table IV). Stephenson and Trim (28) found that *B. coli* deaminates and dephosphorylates adenine triphosphate.

All the bacteria studied in this paper, with the exception of *Ph. campestris*, oxidized lactate at considerably higher rates than they oxidized glucose. Moreover, *A. faecalis*, Strain 2, and *M. freudenreichii*, which did not oxidize glucose, oxidized lactate readily (Table III). Cook and Stephenson (29), working with a

TABLE IV

Effect of Adenine Triphosphate on Oxidation of Glucose by Micrococcus freudenreichii and Alcaligenes faecalis, Strain 2

Amount of glucose, 0 03 mM; amount of adenine triphosphate, 0 01 mM.

Substrate	O_2 consumption per hr.	
	M freudenreichii	*A. faecalis*, Strain 2
	c.mm.	c.mm.
None...................... . .	40 4	26.0
Glucose...................... ...	40 6	30 0
Adenine triphosphate	58 3	205 0
Glucose + adenine triphosphate	74 2	206 0

TABLE V

Metabolism of α-Keto Acids

The oxidation results are given as O_2 uptake in phosphate buffer, pH 7.06, gas phase, air; those for dismutation as CO_2 output in bicarbonate buffer, pH 7.4, gas phase, N_2-CO_2. The figures give c mm. per hour per mg. of dry weight (O_2 uptake without the substrate subtracted).

Bacteria	Pyruvate metabolism		α-Ketogluta-rate
	Oxidation	Dismutation	Oxidation
Ps. aeruginosa......... .	165 0	5 8	7 8
Ph. campestris....... ...	98 2	0	
G. tetragena	44 4	6 8	49 6
S. lutea	48.8	0	7.8
M. piltonensis	175 7	0	0
A. faecalis, Strain 2 .	71 2	6 1	3.8
M. freudenreichii	19 6	0	5.4

strain of *A. faecalis* unable to oxidize glucose, found that the bacteria oxidized lactic, acetic, and formic acids.

All the bacteria studied in this paper oxidized pyruvate at higher rates than they oxidized glucose. Moreover, *A. faecalis*, Strain 2, and *M. freudenreichii*, which did not oxidize glucose,

oxidized pyruvate readily (Table V). They utilized pyruvate very little in the absence of oxygen. Indeed, *S. lutea, M. piltonensis*, and *M. freudenreichii* did not utilize it at all. These observations together with those of Barron and Lyman (30) on the lack of anaerobic metabolism of pyruvate by the red blood cells of the goose may be taken as an indication that the oxidation and the dismutation (anaerobic metabolism) of pyruvate are two different processes. Since both require the presence of diphosphothiamine (Barron and Lyman (30)), which is present in

TABLE VI

Oxidation of Mono- and Polycarboxylic Acids, Alcohols, Amino Acids, and Catechol

Temperature, 38°; pH, 7.06; air as gas phase; amount of substrate, 0.01 mM. The figures give c.mm. of O_2 uptake per hour per mg. of dry weight (O_2 uptake without the substrate subtracted).

Substrate	Ps. aeruginosa	Ph. campestris	G. tetragena	S. lutea	A. faecalis	M. freudenreichii
Formate	48.7	30.0	35.6	34.7	106.0	15.1
Acetate	85.5	118.0	75.3	53.0	12.3	44.4
Propionate	91.9	97.0	67.2	84.2	4.0	42.2
Butyrate	125 3	19.0	91.5	80.0	15.4	42.8
Succinate	23 4	122.5	32.3	100.1	50.4	60.5
Fumarate	17.8	24.4	17.1	83.7	14.0	24.8
Citrate	2.5	8 0	4.3	3.8	10.6	3.1
Ethyl alcohol	58.7	0	183.0	82.1	3.7	3.0
Glycerol	21.8	25.3	4.0	32.0	13.7	8.7
dl-Alanine	90.5	43.7	26.0	39.0	14.7	119.6
l(+)-Glutamate	182.0	17.1	84.0	72.0	22.5	146.5
Catechol	59.1	15.1	14.3	0	0	0

these bacteria, it is possible that there are two proteins, one specific for the oxidation, and the other specific for the dismutation of pyruvate.

It is known that decarboxylation of α-keto acids by yeast is limited to α-ketomonocarboxylic acids (Neuberg and Kerb (31)). McGowan and Peters (32) and Long and Peters (33) have shown that α-ketomonocarboxylic acids are oxidized by chopped brain tissue from pigeons, while α-ketodicarboxylic acids (α-ketoglutaric acid) are not. *M. piltonensis*, which oxidized pyruvate very readily, did not oxidize α-ketoglutarate. In general, the oxida-

tion of α-ketoglutarate was from 3.6 to 21.2 times as slow as the rate of oxidation of pyruvate. Only with *G. tetragena* was the rate of oxidation of both ketocarboxylic acids about the same (Table V).

Oxidation of Polycarboxylic Acids and Effect of These Acids on Rate of Glucose Oxidation—All the bacteria oxidized succinate rapidly; fumarate was oxidized with less speed; and citrate was oxidized very slowly (Table VI). According to von Szent-Györgyi (34) and Krebs and Johnson (35) these acids act as catalysts for the oxidation of glucose in certain animal tissues. The effect of fumarate on the rate of oxidation of glucose by *B. coli* was studied by Califano and Banga (36), who found no catalytic effect. In

<div align="center">TABLE VII</div>

Effect of Fumarate and Citrate on Rate of Glucose Oxidation by Staphylococcus albus and Phytomonas campestris

Temperature, 38°; pH, 7.03; amount of glucose, 0.03 mM; amount of fumarate or citrate, 0.005 mM. Oxidation = O_2 uptake in c.mm. per hour (O_2 uptake without the substrate subtracted).

Substance	Oxidation	
	Staphylococcus albus	Ph. campestris
Glucose	243.8	106.4
Fumarate.........................	331.7	24.4
Glucose + fumarate............	356.9	140.4
Citrate.........................	49.1	8.0
Glucose + citrate..............	253.4	107.4

Staphylococcus albus, the oxygen uptake in the presence of glucose plus fumarate or glucose plus citrate was less than the sum of the O_2 uptakes with each of these substrates when present alone. In *Ph. campestris* (a non-sugar-fermenting bacterium) there was a slight increase in the presence of glucose plus fumarate (7.3 per cent); the oxygen uptake in the presence of glucose plus citrate was less than that expected from an additive effect (Table VII). Therefore, neither fumarate nor citrate acted as catalyst for the oxidation of glucose by these bacteria. Probably these enzymatic, sluggish oxidation-reduction systems (see Barron (37)) act as catalysts for the oxidation of carbohydrates only in those cells where the rate of oxidation (respiration) is regulated by a number of reaction-controlling mechanisms.

Oxidation of Saturated Fatty Acids—Butyrate, propionate, acetate, and formate were readily oxidized by these bacteria (Table VI). Formate was oxidized rapidly to CO_2 and H_2O by all the bacteria studied, *M. freudenreichii* being the only one in which the rate of reaction was slow. Acetate was likewise oxidized to completion by all the bacteria with the exception of *A. faecalis*, for which the slow rate prevented finding the end of the reaction satisfactorily. Propionate was oxidized at about the same rate as acetate, *A. faecalis*, Strain 2, being the only microorganism which oxidized it at a very slow rate. Butyrate was likewise oxidized at about the same speed as the other two acids, except that *Ph. campestris* oxidized it very slowly. This phenomenon is significant in the problem of unity or multiplicity of oxidation enzymes for saturated fatty acids, because, as already stated, *Ph. campestris* oxidized acetate rapidly.

Oxidation of Alcohols—There is some evidence that the oxidation of monoalcohols and that of polyalcohols are performed by different enzyme systems.[1] Among the bacteria studied here, *Ph. campestris* and *G. tetragena* can be presented as examples supporting that view; the former did not oxidize ethyl alcohol, but did oxidize glycerol; the latter oxidized ethyl alcohol at high speed, while it hardly oxidized glycerol at all. *M. freudenreichii* oxidized both alcohols very slowly (Table VI).

Oxidation of Amino Acids—Kendall, Friedemann, and Ishikawa (20) and Webster and Bernheim (38) have shown that *Ps. aeruginosa* oxidizes amino acids; while in some cases only the natural isomers were oxidized, in others (alanine, serine, tyrosine, proline) there was oxidation of both isomers. Because the non-natural isomer of alanine is readily attacked by bacteria, *dl*-alanine was chosen as an example of this group and *l*(+)-glutamic acid as an example of the natural isomers. All the bacteria oxidized these two amino acids beyond the formation of the corresponding keto acid, as shown by measurements of the consumption of oxygen, which in every case was more than 1 mole of O_2 per mole of amino acid (oxidation to the keto acid requires only half a mole of O_2; Table VI).

Oxidation of Catechol—Polyphenols are reversible oxidation-reduction systems in which the rate of oxidation of the reduced

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[1] Barron, E. S. G., unpublished data.

compound, which is slight in the presence of atmospheric oxygen, becomes tremendous in the presence of polyphenol oxidase (Cu-protein). By virtue of their reversibility and their highly positive oxidation-reduction potential they may act as catalysts for cellular respiration. Indeed, Oparin (39) postulated such a rôle in plant respiration. The oxidation of polyphenols by bacteria has not been extensively studied. Roux (40) showed that hydroquinone was oxidized by *B. coli*, and more recently Happold (41) and Yamagutchi (42) found that *Ps. aeruginosa* oxidized polyphenols. In our experiments, *Ps. aeruginosa* oxidized catechol readily; *M. piltonensis* oxidized it half as well; *G. tetragena* and *Ph. cam-pestris* oxidized it slowly; *S. lutea*, *A. faecalis*, and *M. freuden-reichii* did not oxidize it at all (Table VI). Oxidation of polyphenols is not widely observed in bacteria (for example, *Staphylococcus albus* did not oxidize it). Since the velocity of oxidation of the different substrates by these bacteria is in general greater than the velocity of oxidation of catechol, it may be concluded that this system does not normally act as an oxidation catalyst. The oxidation of catechol by these bacteria is completely inhibited by 8-hydroxyquinoline (0.001 M) and salicyl aldoxime (0.001 M), inhibitors of reactions catalyzed by Cu.

Effect of Inhibitors—HCN (0.005 M) inhibited the oxidation of oxidizable substrates in all the bacteria studied in this paper, showing that in the oxidation of these substances a heavy metal catalyst is present. In the experiments in which pyruvate was used as oxidizable substrate, the concentration of pyruvate was 0.012 M, while that of HCN was 0.001 M, to avoid the objection that the inhibition might have been due to cyanohydrin formation. This inhibition is of special interest in the oxidation of hexose monophosphate and *dl*-alanine, because the oxidation of each of these substances by the enzyme systems isolated by Warburg and coworkers (43) is performed without the intervention of a heavy metal catalyst. Whenever the cells contain a heavy metal catalyst—the cytochrome system in these bacteria—this catalyst seems to act as the last acceptor of electrons before molecular oxygen becomes involved in the reaction. This holds true not only for the oxidation of hexose monophosphate and *dl*-alanine, but also for the oxidations of lactate, pyruvate, and glycerol, which proceed without iron porphyrin catalysis in anaerobic

bacteria but need this catalyst in the bacteria under study (Table VIII).

After iodoacetic acid was introduced as an inhibitor of glycolysis, a number of papers were published, showing that it may act also as an inhibitor of oxidation (Dixon (44)). The oxidation of hexose monophosphate and that of ethyl alcohol by a partially purified enzyme system was inhibited by $BrCH_2COOH$ (von Euler and Adler (45)). CH_2ICOOH was found to inhibit the oxidation of triose phosphate (Rapkine (46)). Iodoacetic acid was a general

TABLE VIII

Effect of HCN (0.005 M) on Oxidations Produced by Bacteria

The results represent the per cent of inhibition.

Substrate	M. freuden-reichii	S. lutea	Ps. aerugi-nosa	A. faecalis, Strain 2	G. tetra-gena
None..............	79.4	41.0	80.0	87.4	32.6
Glucose............		98.0	94.5		86.3
Hexose diphosphate	86.0	92.0	91.5	89.0	88.0
" monophos-					
phate............	79.7	95.0	92.0	90.0	89.0
Lactate	88.0	Complete	96.0	97.4	99.5
Pyruvate	94.7	90.7	76.0	80.4	64.0
Butyrate..........	90.2	Complete	94.7	92.0	Complete
Propionate........	91.4	"	95.0	Complete	"
Acetate............	Complete	99.3	92.2	"	"
Formate...........	"	93.0	84.5	"	84.4
Ethyl alcohol	"	98.9	92.0	"	Complete
Glycerol...........	"	86.7	Complete	"	"
dl-Alanine.........	93.5	96.1	"	"	"
l(+)-Glutamate....	89.5	94.7	96.5	"	"
Succinate..........	97.3	98.4	Complete	"	"
Fumarate..........	Complete	94.8	"	"	"

inhibitor of oxidations produced by the bacteria under study. At a concentration of 0.005 M, it inhibited to a large extent the oxidation of all substrates with few exceptions. (Among the exceptions must be mentioned the oxidation of fumarate, which was inhibited only to a small degree in *M. freudenreichii*, *Ps. aeruginosa*, and *G. tetragena* (Table IX)). It is known (Dickens (47), Rapkine (48)) that iodoacetic acid reacts readily with —SH derivatives like cysteine, glutathione, and the —SH groups of proteins, as well as with the NH_2 group of amino acids (Michaelis and Schubert (49)), although the latter reaction requires conditions

unlikely to be found in biological reactions (high temperature and alkalinity). If iodoacetic acid acts by combining with the —SH groups of the protein component of the oxidation enzymes (Rapkine (16)), there is the possibility that in these bacteria the activating proteins of the oxidation enzymes for all the substances studied contain —SH groups, the presence of which is essential for their activity.

TABLE IX

Effect of CH₂ICOOH on Oxidations Produced by Bacteria

The results represent the per cent of inhibition at different CH₂ICOOH concentrations.

Substrate	M. freuden-reichii		S lutea	Ps. aeruginosa		A faecalis, Strain 2		G. tetragera
	0 005 M	0 001 M	0 005 M	0 005 M	0 001 M	0 005 M	0 001 M	0 001 M
None	None	None	51 0	78 0	None	40 0	None	None
Glucose			57 4	75 0	41 6	80 0	72 0	72.0
Hexose diphosphate	68.0		83.0	80 6	None			
" monophosphate	54.0		60.0	70.0	"	84.0		
Lactate	64 0		47 5	79 0	50 0	66 0	51 0	64.0
Pyruvate	83 0		73.4			*	89 0	74.0
Butyrate .	75 0	67 5	96 4	86.4	47 3			*
Propionate	88.5	79.0	*	90.6	85 0		*	31.0
Acetate·..	68 7	31.3	97.1	92 2	87 0	*	*	
Formate	53 0	None	*	75.0	65.0	94 0	None	63.0
Ethyl alcohol	34 0		89.7	84 0	39 0		*	5 0
Glycerol	35 0		None	74.0	15 0		*	
dl-Alanine .. .	50 3	42 5	84.3	88.0	33 0			9.0
l(+)-Glutamate ...	54 3	25.2	58 8	84.5			72.0	34 0
Succinate..........	59 8	31.8	96 7	89.4	None		96.0	None
Fumarate..........	18 0	18 0	94 2	45 0	"			"

* Complete inhibition.

SUMMARY

The bacteria studied here demonstrate that glucose can be oxidized before previous fermentation, thus distinguishing clearly the fermentation process from the oxidation process. Of this group of non-glucose-fermenting bacteria, there were only two unable to oxidize glucose (*Micrococcus freudenreichii* and *Alcaligenes faecalis*, Strain 2). Because these bacteria did oxidize phosphorylated hexose (hexose monophosphate and diphosphate)

as well as a number of saturated fatty acids, hydroxy acids, keto acids, amino acids, and alcohols, it may be concluded that failure to oxidize glucose is due to lack of phosphorylation.   Phosphorylation occurs in the presence of a protein (phosphorylase) and adenine triphosphate.   The failure to induce phosphorylation of glucose, and hence its oxidation, by adding adenine triphosphate is an indication that lack of phosphorylation is due to lack of the protein of the phosphorylating enzyme.   The oxidation of glucose was generally accompanied by CO_2 production, the R.Q. being about 1; *Micrococcus piltonensis* oxidized glucose without CO_2 production.   With *Pseudomonas aeruginosa* there was complete oxidation of glucose, for about 6 moles of O_2 were taken up per mole of glucose.

The complete inhibition of the oxidation of hexose monophosphate and unnatural amino acids by HCN, a phenomenon previously observed for the latter substrates by Webster and Bernheim (38), is presented here in support of the view that the components of individual oxidation systems are not the same but vary in different cells.   Thus, in these bacteria hexose monophosphate and unnatural amino acids are oxidized through the mediation of the cytochrome system or some other heavy metal catalyst, and not through the direct reaction between alloxazine and molecular oxygen, as happens in the enzyme systems isolated by Warburg and his coworkers.   This concept of varied mechanisms of oxidation of individual substrates, which will be further strengthened with experiments on bacteria lacking the cytochrome system, must always be kept in mind to check rash generalizations from work done on the isolation of the components of oxidation enzymes.

The experiments on CH_2ICOOH inhibition of biological oxidations were performed to show that this substance is not a specific inhibitor of the anaerobic splitting of glucose.   Almost all oxidations studied in this paper were inhibited by CH_2ICOOH. Whether this inhibition demonstrates that the protein components of all these oxidation enzymes contain in these bacteria —SH active groups which become inactive on combination with CH_2ICOOH is still a question.   Both the inhibition by HCN of all oxidations and the presence of cytochromes are offered in favor of the view that the cytochrome system is a necessary component of all the oxidation enzymes in these bacteria.   An excep-

tion may be the oxidation of catechol by some of the bacteria (*Pseudomonas aeruginosa*, *Phytomonas campestris*, *Gaffkya tetragena*), for the oxidation was completely inhibited by 8-hydroxyquinoline and by salicyl aldoxime which forms a complex compound with copper.

The same distinction between aerobic and anaerobic metabolism (oxidation and fermentation) has also been demonstrated with pyruvate. As a rule, cells possessing fermentation enzymes are able to split pyruvate in the absence of oxygen; it may now be added that cells unable to ferment glucose are also unable to split pyruvate in the absence of oxygen. Of the seven bacteria studied in this paper, four failed completely to split pyruvate in the absence of oxygen (*Phytomonas campestris*, *Sarcina lutea*, *Micrococcus pittonensis*, and *Micrococcus freudenreichii*); the other three split it at extremely slow rates compared with the rates of oxidation. Since there are cells able to oxidize pyruvate while unable to ferment it, although the necessary coenzymes are present, it may be postulated that there are two different activating proteins, one for the oxidation, the other for the fermentation of pyruvate.

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THE SYNTHESIS OF S-(β-AMINO-β-CARBOXYETHYL)-HOMOCYSTEINE

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Attention has recently been focused on the mixed thio ether of α-alanine and α-aminobutyric acid

$$\underset{\displaystyle HOOC- CH- CH_2- CH_2- S- CH_2- CH- COOH}{\overset{\displaystyle NH_2 \qquad\qquad\qquad\qquad NH_2}{\mid \qquad\qquad\qquad\qquad\qquad\mid}}$$
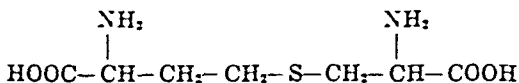
which may be regarded as either a derivative of cysteine or homocysteine. This compound, S-(β-amino-β-carboxyethyl)-homocysteine, was suggested several years ago by Brand and coworkers (1) as a possible intermediate in the *in vivo* conversion of methionine to cystine. Toennies (2) has, in addition, recently drawn attention to the methyl sulfonium base of this mixed thio ether as another possible intermediate in the conversion just mentioned. Additional interest in the mixed thio ether also arises from the fact that Horn and Jones (3) have isolated, from a plant grown on seleniferous soil, a substance which appears to be an isomorphous combination of this thio ether and 2 molecules of the selenium analogue.

The first mention of such a thio ether structure in connection with biological material was in the work of Küster and Irion (4) in which a substance, believed to have this structure, was isolated from a hydrolysate of wool. The wool, however, had been treated with 3 per cent sodium sulfide solution for 20 days before the hydrolysis with acid and it is indeed possible that the compound may have arisen during this treatment, since the isolation could not be repeated by them. The compound isolated had an empirical formula which corresponded to that of such a thio

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ether, but the evidence in proof of the suggested structure was admittedly very inadequate.

With the purpose in mind of testing the possible rôle of the thio ether in sulfur metabolism, its synthesis was undertaken. Homocysteine, prepared by the method of Riegel and du Vigneaud (5), and α-amino-β-chloropropionic acid, prepared from serine by a modification of the method of Fischer and Raske (6), were combined in strongly alkaline solution to produce the desired S-(β-amino-β-carboxyethyl)-homocysteine. Subsequent experiments showed that a better over-all yield from serine could be obtained by using directly the methyl α-amino-β-chloropropionate hydrochloride without conversion to and isolation of the free acid.

EXPERIMENTAL

S-(β-Amino-β-Carboxyethyl)-Homocysteine—For the preparation of large quantities of methyl α-amino-β-chloropropionate hydrochloride a modification of the method of Fischer and Raske (6) was used. 400 cc. of acetyl chloride were placed in a 1 liter, 3-necked flask which was equipped with a stirrer and gas inlet tube. The flask was cooled in an ice bath, and dry HCl was passed into the solution for a short time. The introduction of HCl was continued, and 50 gm. of finely pulverized serine methyl ester hydrochloride (7) were added. The introduction of HCl was stopped and 75 gm. of finely pulverized PCl_5 were added in small portions over a period of 20 minutes. The solids dissolved slowly and some flocculent precipitate began to appear. The stirring was continued at room temperature for 45 minutes and then 300 cc. of high boiling petroleum ether were added in 25 to 50 cc. portions during a period of 30 minutes. It is of advantage to add seed crystals during the addition of the petroleum ether. The precipitate, which was collected on a large sintered glass funnel, was washed twice by suspension in 400 cc. of high boiling petroleum ether and finally by suspension in low boiling petroleum ether. The product was dried *in vacuo* and amounted to 47 to 52 gm., which represents 85 to 90 per cent of the theoretical yield. This product decomposed from 130–135° and is satisfactory for use in the subsequent steps. The recrystallized product decomposed at 132–135° which agreed with the decomposition point reported by Fischer and Raske (6).

The condensation of the methyl α-amino-β-chloropropionate with homocysteine was carried out in the following way. To 20 gm. of homocysteine in 25 cc. of oxygen-free water were added 35 gm. of KOH in 40 cc. of water. An atmosphere of nitrogen was maintained in contact with the solution. With vigorous stirring 27 gm. of the methyl α-amino-β-chloropropionate were added during a 20 minute period. The temperature was maintained at 50° by occasional cooling. The stirring was then continued at room temperature for 3 hours. The mixture was then diluted with 3 volumes of water and, after the solution had been cooled in an ice bath, it was neutralized with concentrated HI. It was then made alkaline to litmus with NH₄OH, a drop of FeCl₃ was added, and the solution was gently aerated to oxidize the homocysteine present. 5 gm. of homocystine were removed by filtration and the solution was concentrated to a volume of 150 cc. The solution was again filtered to remove a further small amount of homocystine and then was filtered through a layer of charcoal. 3 volumes of alcohol were added, and the precipitate, which was deposited after standing overnight in the refrigerator, amounted to 12 gm. This precipitate was dissolved in 400 cc. of water. It was filtered to remove a small amount of homocystine and an equal volume of alcohol was added to the filtrate. The product which was obtained was twice recrystallized by solution in water and precipitation by alcohol. After the material was washed by suspension in alcohol and then in ether, 6.1 gm. of the compound were obtained, representing 18 per cent of the theoretical yield. In addition, 28 per cent of the homocysteine used was recovered as homocystine. The compound decomposed at 270° and had the following composition.

$C_7H_{14}O_4N_2S$. Calculated. N 12.58, S 14.39
Found. " 12.42, " 14.58

The synthetic product possessed properties which might be expected of such a thio ether. A negative nitroprusside test was obtained both before and after treatment with sodium cyanide. The nitrogen was shown to be entirely present as amino nitrogen by the Van Slyke procedure. Treatment of the compound with concentrated HI resulted in considerable decomposition but small amounts of homocysteine thiolactone and cysteine were shown to be present in the reaction mixture, thus demonstrating the

presence of these moieties in the synthetic compound. The synthetic compound is very likely a mixture of the two inactive modifications which are theoretically possible due to the presence of 2 asymmetric carbon atoms. No evidence of fractionation was observed during the isolation of the compound.

The synthesis was also carried out with the sodium salt of homocysteine which was obtained by the reduction of S-benzylhomocysteine by Na in liquid NH_3. This was done to circumvent the necessity for isolating the homocysteine. 60 gm. of benzylhomocysteine were dissolved in liquid NH_3 and reduced (5). The NH_3 was distilled and the residual NH_3 removed by evacuation. The residue was dissolved in 60 cc. of cold oxygen-free water and 60 gm. of KOH in 50 cc. of water were added, followed by 45 gm. of methyl α-amino-β-chloropropionate hydrochloride. The product was isolated by the procedure described above and amounted to 8 to 9 gm., representing 13 to 15 per cent of the theoretical yield based on the benzylhomocysteine used.

N,N'-Dicarbobenzoxy-S-(β-Amino-β-Carboxyethyl)-Homocysteine —250 mg. of the S-(β-amino-β-carboxyethyl)-homocysteine were dissolved in 0.5 cc. of water and 0.6 gm. of carbobenzoxy chloride and 1.0 cc. of 4 N NaOH were added slowly with vigorous shaking over a period of 30 minutes. The alkaline solution was extracted with ether, acidified with H_2SO_4, and was exhaustively extracted with ether. The ether extract was evaporated to dryness and the residue was dissolved in ethyl acetate and precipitated by petroleum ether. The viscous oil was then rubbed up with three portions of petroleum ether and was finally dissolved in $CHCl_3$ and precipitated by gradual addition of petroleum ether. The product melted at 108–111° with slow decomposition.

$C_{23}H_{26}O_8N_2S$.   Calculated.   C 56.31, H 5.34, N 5.71, S 6.53
                              Found.   " 55.90, " 5.41, " 5.70, " 6.72

SUMMARY

The synthesis is presented of the thio ether, S-(β-amino-β-carboxyethyl)-homocysteine, which was reported by Küster and Irion to have been isolated from sodium sulfide-treated wool

and by Horn and Jones from a plant grown on seleniferous soil. The synthesis was brought about through the reaction of α-amino-β-chloropropionic acid with homocysteine in strongly alkaline solution.

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A SIMPLE, ACCURATE METHOD OF ESTIMATING CARBON MONOXIDE IN BLOOD

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There are two chief manometric methods for estimating CO in blood; *viz.*, that due to Van Slyke and Neill (4) and that due to Sendroy and Liu (3). In both of these the first stage is the liberation of all the O_2, CO_2, CO, and N_2 of the blood by shaking *in vacuo* with acid ferricyanide. In the Van Slyke-Neill method the O_2 and CO_2 are then absorbed with alkaline hyposulfite, leaving a residue of CO and N_2. The amount of N_2 is calculated from its solubility in blood and thence by subtraction the CO content is obtained. Owing to the blank correction for dissolved N_2 and also for the slight reabsorption of CO by the reduced hemoglobin, which is formed after the addition of the hyposulfite, the method is only accurate to 0.2 volume per cent CO, but this is good enough for many purposes.

In the Sendroy-Liu method the extracted gas is transferred to a Hempel pipette containing alkaline pyrogallol and the O_2 and CO_2 therein are absorbed. The gas is then returned to the usual Van Slyke-Neill chamber and the CO estimated by absorption with cuprous chloride. The method is more difficult and much more elaborate than the Van Slyke-Neill method, but gives as high an accuracy as is ever likely to be needed.

The method now to be described is as simple as the Van Slyke-Neill method but approaches the Sendroy-Liu method in accuracy. It has a further advantage, not possessed by any previous method; namely, that it can be used in Barcroft differential manometers as well as in the Van Slyke-Neill manometers, and so may be of service in laboratories which happen to be better equipped with the former.

CO Estimation in Van Slyke-Neill Manometer

Principle of Method—The blood is laked and shaken in the Van Slyke-Neill chamber with a sodium glycinate-hyposulfite (pH about 10) mixture in the dark for 3 minutes. The O_2 and CO_2 remain in solution chemically bound, but practically all the dissolved N_2 is extracted into the vacuum. The affinity of CO for reduced hemoglobin is, under these conditions, so high that no appreciable amount is normally liberated.[1] The extracted N_2 is quantitatively ejected and excess K_3FeCy_6 drawn into the chamber, which is then shaken until the whole of the COHb is dissociated. The only gas evolved is CO, which can therefore be measured accurately without use of cuprous chloride or other special absorbing reagents. The manipulative procedure is thus exceptionally easy.

Reagents Required—

Saponin solution. 1 gm. of Merck's saponin is dissolved in 100 cc. of water.

Glycinate-hyposulfite solution. This is a 1.0 M solution of glycine in 0.9 M NaOH, containing 2 per cent hyposulfite. 50 cc. of it are enough for a day's work and are prepared freshly as follows: 3.75 gm. of glycine (analytical reagent) are dissolved in 45 cc. of carbonate-free 1 N NaOH in a 50 cc. stoppered cylinder. 1 gm. of $Na_2S_2O_4$ is added, the stopper inverted, and the solid dissolved with minimal shaking. The volume is then made up to 50 cc. and the solution at once transferred to a burette under paraffin oil. It should only be used on the day of preparation.

Potassium ferricyanide solution, 32 per cent. This solution is deaerated in a vacuous Barcroft tonometer rather than in the Van Slyke-Neill chamber, since strong K_3FeCy_6 oxidizes mercury rather readily. The solution is stored in a burette.

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[1] The following considerations indicate that no significant proportion of CO from the COHb will escape into the gas phase during the 3 minute extraction. At least an hour's shaking would be needed for equilibrium between the CO in the liquid and gas phases and even then inspection of the COHb dissociation curve shows that the volume of CO liberated into the gas phase should not exceed 0.5 c.mm., if the percentage saturation of the hemoglobin lies within the range for which the present method is intended; *viz.*, 0 to 40 per cent. In 3 minutes the amount of CO liberated should thus be utterly negligible.

Procedure

4 drops of octyl alcohol are drawn into the Van Slyke-Neill chamber and 2 cc. of saponin solution are then placed in the cup. 2 cc. of the blood are then drawn into the chamber, followed by the 2 cc. of saponin. The blood and saponin are mixed in the chamber, and, after 1 minute for laking to be complete, 1.5 cc. of the glycinate-hyposulfite solution are drawn into the chamber which is then covered by black paper (or tin-foil). The mercury is lowered to the 50 cc. mark and the mixed solution shaken *in vacuo* for 3 minutes. The black paper is then removed and the extracted N_2 is quantitatively ejected. 0.5 cc. of deaerated ferricyanide is drawn into the chamber, the mercury lowered to the 50 cc. mark, and the chamber shaken for 10 minutes. The pressure is measured at the 2.0 or 0.5 cc. mark. The solution is then reevacuated and shaken for a further 5 minute period to check that the endpoint has been reached (pressure reading p_2). This is so if $p_2 - p_1$ ≯ 0.3 mm. of Hg, as should be the case if the temperature is 22° or over.

The gas is then quantitatively ejected and a final pressure reading taken, p_3.

The CO content of the blood = $(p_3 - p_2 - c)$ × constant.

The c correction is obtained by a blank with water in place of blood. It should not exceed 0.5 mm. of Hg, being mainly due to dissolved N_2 which is not quite completely extracted during the first shaking. The value of the constant, correct to within 0.1 per cent, is given by the most right-hand column of Table 30 of Peters and Van Slyke (2). Thus at 23° the constant = 0.1215, and if $(p_3 - p_2 - c) = 15$ mm. of Hg, the CO content = 1.83 volumes per cent. The whole procedure takes about 30 minutes, but in nearly half of this time the observer is free to undertake some other task. At temperatures below 20° longer shaking periods are necessary, owing to the high temperature coefficient of the rate of dissociation of COHb. The method has only been used over the range 0 to 30 per cent COHb saturation, since this is of most practical importance in blood volume determinations and in estimations of relatively small degrees of CO poisoning. It gives equally good results with human, pig, and ox blood, and in the presence of heparin or oxalate as anticoagulant. It works

also with defibrinated blood with or without 1 per cent boric acid as preservative.

Accuracy—The maximum discrepancy between duplicate values of $(p_3 - p_2 - c)$ was found to be 0.6 mm. of Hg, while the average was 0.4 mm. of Hg. The latter is near to the limits of accuracy of the Van Slyke-Neill apparatus, and with 2 cc. blood samples corresponds to a figure of 0.05 volume per cent CO or to 0.25 per cent saturation with CO. This precision is of the same order as is claimed in the Sendroy-Liu method, and is as great as is needed for practical purposes. For this reason no tests have yet been made with 5 cc. blood samples, though presumably the same proportionate increase in precision would be obtained as is found in the Sendroy-Liu method.

A further check on the accuracy of the method is provided by the comparisons with other methods which are summarized in Table II. This will be discussed later.

CO Estimation in Barcroft Manometer

Principle of Method and Reagents Required—The principle is exactly the same as in the Van Slyke-Neill manometer except that the CO is liberated into a gas phase consisting of N_2 at atmospheric pressure instead of into a vacuum. The only additional solution required is 1 per cent NaCl, the other reagents being identical with those used in the Van Slyke method, though there is no need in this case to deaerate the ferricyanide. A tank of compressed N_2 and a 5 liter pressure-equalizing bottle are also required.

Procedure

2 cc. of saponin solution and 2 cc. of 1 per cent NaCl are run into the left-hand bottle, Bottle L, of the Barcroft apparatus; then 2 cc. of saponin solution and 2 cc. of the blood sample are run into Bottle R, the right-hand bottle. Bottle R is then mixed by two quick shakes, no appreciable COHb dissociation being caused thereby. 1.5 cc. of the glycine-hyposulfite solution are next run below the surface of the liquid in Bottle L, and then similarly in Bottle R. A Keilin dangling tube containing 0.5 cc. of 32 per cent ferricyanide is then loaded into Bottles L and R respectively and the two bottles are at once fixed to the Barcroft manometer, the

taps and joints of which must be thoroughly gas-tight. The manometer is connected to a water filter pump and the whole apparatus evacuated to 0.1 atmosphere and washed thrice with N_2 gas. Finally the bottles and their connections to the gage are left filled with N_2 at atmospheric pressure. The manometer is then at once mounted in a bath and shaken at about 120 round trips per minute, temperature 20–25°. The whole filling process should take about 6 minutes and care should be taken not to expose the hyposulfite unduly to the air, nor to allow the N_2 to enter the manometers too suddenly after the pressure in them has been reduced, as otherwise the gage liquid may be blown over. After 10 to 15 minutes shaking, the gage readings generally become steady, temperature equilibration and absorption of traces of O_2 in the commercial N_2 both being complete in this time. The manometer is then quickly lifted out of its stand, and the Keilin cups dislodged by a sharp tap so that the ferricyanide is mixed with the solutions in each bottle. The manometer is at once returned to the shaker and shaking continued until there is no further evolution of gas. The time required for this is usually 25 to 30 minutes at 22°. The CO content of the blood is then calculated with the aid of the constant of the apparatus determined by the mercury weighing method as described by Dixon (1). Table I shows a typical protocol of quadruplicate determinations on a sample of ox blood containing 15 per cent COHb.

Each pair of columns gives the readings of the left and right limbs of the manometer, the serial number of which is at the head. The letter K shows the stage at which the contents of the Keilin cups are shaken into the blood. In the case of Manometer BR the shaking was continued for 45 minutes after the end-point had been reached, in order to make sure there was no drift. In a few cases shaking was continued as long as 24 hours, and even then drifts of no more than 1.0 mm. were observed, which might very well be attributed to decomposition of the solutions or to very slight leakage. The only instance in which a satisfactory end-point was not obtained was when the hyposulfite used appeared to contain some sulfide. Here the results were worthless, for much less than the expected volume of CO was evolved, and then followed a steady absorption of some gas. Care must therefore

be taken to use a good sample of $Na_2S_2O_4$.   That used in the present work was supplied by The British Drug Houses, Ltd., and the solid was kept out of contact with air as far as possible.

TABLE I

Barcroft Method for Estimating CO in Blood

A 2 cc. sample of defibrinated ox blood treated with 1 per cent boric acid as preservative.

Time	Manometer BR		Manometer B55		Manometer B54		Manometer B66	
	Left	Right	Left	Right	Left	Right	Left	Right
min.								
0	101.2	109.5						
5	101.4	109.3						
10	101.8	109.0	100.9	100.9				
15	101.9	109.0	100.6	101.3				
	K •							
20	93.9	117.0	100.6	101.3	939	968		
			K					
25	90.7	120.2	109.0	93.0	958	951		
30	89.9	120.9	111.5	91.2	961	950	710	853
35	89.9	120.9	111.7	90.5	961	950	680	1091
					K			
40	89.7	121.0	111.7	90.3	1010	902	680	1093
45	89.7	121.0	111.7	90.3	1060	852	680	1094
							K	
50	89.7	121.0	111.7	90.2	1068	845	752	1026
55	89.6	121.1	111.7	90.3	1070	841	786	996
60	89.6	121.1	111.7	90.3	1072	840	789	990
65	89.6	121.1	111.7	90.0	1072	840	789	990
85	89.6	121.1						
CO evolved, *mm. gage fluid*	24.4		22.4		22.1		21.3	
Constant.......	242		255		265		266	
CO, *vol. %*.....	2.95		2.86		2.93		2.84	

Average = 2.90 volumes per cent.

CO content by Van Slyke method = 2.91 volumes per cent.

* *K* represents the stage at which the contents of the Keilin cups are shaken into the blood.

The method has been tested under the same range of conditions as the Van Slyke method (see above).

Accuracy and Applicability—In some thirty duplicates, there was only one case in which the discrepancy was greater than 0.2 volume per cent CO (*i.e.*, 1 per cent COHb saturation). The average discrepancy was 0.12 volume per cent CO (*i.e.*, 0.6 per cent COHb). The method is thus only about one-third as accurate as the Van Slyke method described above. Since no Barcroft method has hitherto been available for CO estimations in blood, the present one was thoroughly tested against the Van Slyke method of this paper. The maximum discrepancy between these two methods was 0.14 volume per cent CO(=0.7 per cent COHb), and the average discrepancy 0.06 volume per cent CO(= 0.3 per cent COHb). The mean of several Barcroft determinations on one sample agrees extraordinarily closely with the results obtained by the Van Slyke method (Table II).

The time taken for a single determination is 45 to 50 minutes, but most of this time is spent in shaking; in a series of determinations the method is much more expeditious, for as Table I shows it is possible to load the contents of the Barcroft apparatus into the shaker at the rate of one per 10 minutes and to complete ten determinations within a space of 2 hours. The Barcroft method is thus amply accurate for measuring small amounts of CO in the blood of subjects whose occupation renders them liable to CO poisoning. It is indeed particularly suitable for application on a large scale, since the contents of twelve or more Barcroft manometers can easily be shaken simultaneously and data on a large number of individuals subject to a given condition can thus be quickly and readily secured. For blood volume determinations with small amounts of CO it is perhaps, at best, only just exact enough, though even here it offers one compensatory advantage; namely, that if the blood from the subject before breathing CO is placed in Bottle L (in place of 1 per cent NaCl) and the blood after breathing CO is placed in Bottle R, the manometer reading gives *directly* the increase in CO content of the blood as a result of inspiring the measured volume of CO. With the Van Slyke methods two separate CO determinations are necessary, and the CO increase of the blood obtained therefrom by subtracting the first from the second thus leads to some loss in accuracy.

The success of the method with the Barcroft manometers suggests that it should be equally feasible with the Warburg manometric technique, but owing to lack of time this has not yet been tried.

Comparison with Other Methods—Table II summarizes the comparisons of the methods of the present paper with each other and with previous methods. The results are expressed in volumes per cent of CO.

In the mixture method 4.7 cc. of CO-saturated blood were mixed with 32.6 cc. of aerated blood, and the CO content of the mixture calculated from the combining capacity of the blood and the CO content of the aerated blood.

TABLE II

Comparison of Volumes Per Cent of CO by Present and Previous Methods

Van Slyke method of this paper	Barcroft method of this paper	Mixture method	Method of Van Slyke and Neill
2.08(1)	2.05(9)		
2.90(1)	2.90(4)		
3.09(1)	3.08(7)		
4.50(1)	4.60(1)		4.49(1)
4.68(1)	4.82(1)		
4.52(1)	4.58(1)		
2.66(1)		2.55(1)	
	2.25(1)		2.30(1)
7.10			7.20*(1)

The figures in parentheses represent the number of determinations.

* This sample of blood was first freed of dissolved N_2 before being treated with CO, thus eliminating the uncertainty in the Van Slyke-Neill method due to the correction for dissolved N_2.

The results of the present paper not only agree satisfactorily with one another, but also, within experimental error, with the results of the previous methods.

SUMMARY

Normally blood contains four gases; *viz.*, O_2, CO_2, N_2, and CO. The principle of the method is to bind the O_2 and CO_2 by mixing the blood solutions with an alkaline glycinate-hyposulfite mixture, and at the same time to evolve the N_2 into the gas phase. The only remaining gas in solution is then CO, which is subsequently liberated by shaking with neutral ferricyanide solution. The

technique can be carried out both in the Van Slyke-Neill apparatus and in the Barcroft differential manometer. The two apparatus give extremely concordant results both with each other and with alternative methods of estimating CO in blood.

In the Van Slyke-Neill apparatus the method gives results of the same order of precision as the Sendroy-Liu method (*viz.* ± 0.025 volume per cent of CO with a 2 cc. blood sample); it is, however, very much simpler, being indeed easier than the Van Slyke-Neill method, since no special gas-absorbing agents are required.

The results with the Barcroft apparatus are not quite so accurate, but this technique is especially convenient when a large number of estimations are required in the course of 1 day.

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INFLUENCE OF DIFFERENT NUTRITIONAL CONDITIONS UPON THE LEVEL OF ATTAINMENT IN THE NORMAL INCREASE OF CALCIUM IN THE GROWING BODY*

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In an earlier paper (1) it was shown that the percentage of calcium in the body at all ages from late infancy (28 days) to full maturity (1 year) is notably greater in rats on a family diet containing 0.64 per cent of calcium in the dry food mixture than in those whose diet is exactly similar except that the calcium content is only 0.20 per cent. Moreover, the *rate* at which normally developing individuals approach the full adult percentage of body calcium that their particular diet permits is more rapid at the higher level of calcium intake. When the diet is further enriched with calcium salts to bring its total calcium content to 0.80 per cent of the dry food, the rate of body calcification, but not the final percentage, is augmented still further.

The investigation here reported takes as its starting point a diet which is higher in calcium content (0.35 per cent) and better proportioned with regard to other nutritional factors as well. This basal ration is the Diet B (Laboratory No. 13) of earlier papers (2) and consists of one-third dried whole milk and two-thirds ground whole wheat (with sodium chloride and distilled water), as contrasted with one-sixth dried whole milk and five-sixths whole wheat in the basal diet of the previous experiments of Lanford and Sherman (1).

Thus the present experiments deal with *relatively* smaller increases in the level of calcium intake; and these are superimposed upon a basal diet of slightly higher protein content and considerably richer in riboflavin and in vitamin A.

The animals used in the present investigation were albino rats of the Osborne-Mendel strain, pure bred in our laboratory for over 50 generations, and those analyzed as representative of the effects of a given diet were in all cases of second and third generations on the particular diet; that is, the family had subsisted upon the stated diet since the infancy of the parents or of the grandparents of the individuals analyzed.

In the selection of those analyzed at 28 days of age the plan was that, of the twenty-five animals of each sex taken from families on each diet, ten should be early offspring of as many mothers, ten others should be later offspring of the same mothers, and the remaining five should be representative third generation young chosen at random.

For analysis at later ages, sixteen males and sixteen females, likewise of the second or a later generation on the family diet, were continued on their respective diets until 60 days of age, when they were killed for calcium determination; others were kept on the diet until 90 days of age. Sixteen males, but fewer females, were similarly reared to 180 days for analysis; and twenty-five males were reared to 1 year of age.

When the animals attained the specified age, they were killed by chloroform, the gastrointestinal tract removed and the weight of its contents determined, the remaining carcass[1] cremated, and the ash dissolved and analyzed for calcium by the volumetric (or, in a few cases, the gravimetric) modification of the McCrudden method as previously applied in this laboratory (1, 3).

The diets whose effects were thus studied were *Diet 13*, consisting of one-third dried whole milk and two-thirds ground whole wheat with sodium chloride added in the proportion of 2 per cent of the weight of the wheat, this dry food mixture containing 0.34 to 0.35 per cent of calcium; *Diet 132*, like the preceding except that its calcium content was brought to 0.48 per cent by the addition of calcium carbonate; and *Diet 133*, like both the preceding

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[1] Previous work in this laboratory has shown that the thoroughly cleansed gastrointestinal tract of a rat does not contain a weighable amount of calcium. Hence its omission in cremation of the animal for calcium determination involves no appreciable loss and avoids the possibility of contamination with minute particles of undigested food.

except that by further addition of calcium carbonate the calcium content was increased to 0.64 per cent. The animals had access to their respective diets and to distilled water at all times.

DISCUSSION

In Tables I and II are given, for males and unmated females, respectively, the average values for (1) the net body weight (*i.e.*, the gross body weight less the weight of the gastrointestinal contents), (2) the weight of body calcium, and (3) the latter value as percentage of the former.

For the 28, 60, 90, and 180 day-old groups, averages are given which include all of the animals analyzed. Then, if in any group an individual showed very marked deviation in calcium content from others analyzed or if the range and distribution of body weights within the group did not appear to be representative when compared with much larger numbers of the general population, a second average is given which excludes the exceptional individuals. Of the year-old animals, a considerable number which had suffered a substantial loss from their maximum body weight were excluded before the average shown in Table I was drawn.

Attainments in Normal Calcification on Basal Diet of Earlier Study Compared with Nutritionally Superior Basal Diet of Experiments Here Reported (Diets 16 and 13)

Earlier papers from this laboratory (2) have shown the far reaching nutritional superiority of the diet containing one-third, compared with that containing one-sixth, whole milk powder in the dry food mixture. The data here reported enable us now to compare quantitatively (and, we think, conclusively) the calcium aspect of the body composition, at different ages, in otherwise parallel animals on these two dietaries. On the basis of a statistical comparison of the data of Tables I and II with those tabulated in our previous paper (1), it may be stated briefly that at 1 month, 6 months, and 1 year of age, the differences in *percentage* of body calcium (resulting from these two diets) are so small that any of them standing alone would probably not be regarded as statistically significant. At 2 and 3 months of age, however, there are unquestionably significant differences in favor of the diet which, with the higher proportion of milk, contains 0.35 per cent of

calcium in the dry food, and is also somewhat richer in protein and considerably richer in vitamin A and riboflavin. The per-

TABLE I

Calcium Content of Male Rats

Age	Diet No.	Ca in diet	No. of cases	Net weight		Calcium content				
						Total		Per cent of net weight	
				Mean	C.v.*	Mean ± p.e.	C.v.*	Mean ± p.e.	C.v.*
days		*per cent*		*gm.*		*gm.*			
28	13	0.35	25	43.3	10.0	0.318 ± 0.004	10.3	0.734 ± 0.004	3.8
			23†	43.4	10.4	0.321 ± 0.004	10.1	0.740 ± 0.003	2.9
	132	0.48	28	43.3	10.6	0.345 ± 0.004	10.2	0.799 ± 0.004	4.4
	133	0.64	26	41.8	9.6	0.357 ± 0.004	9.6	0.854 ± 0.005	4.7
60	13	0.35	16	143.9	12.0	1.132 ± 0.020	10.6	0.789 ± 0.005	3.8
			15‡	141.8	11.0	1.117 ± 0.020	10.5	0.790 ± 0.005	3.9
	132	0.48	16	150.7	12.4	1.293 ± 0.023	10.8	0.860 ± 0.005	3.2
			11‡	142.8	12.2	1.233 ± 0.024	10.0	0.866 ± 0.006	3.5
	133	0.64	16	134.9	19.0	1.247 ± 0.032	15.4	0.930 ± 0.007	4.3
			15‡	131.6	15.4	1.223 ± 0.030	14.2	0.934 ± 0.006	4.0
90	13	0.35	16	221.8	8.1	2.013 ± 0.030	8.8	0.908 ± 0.007	4.6
			14†, ‡	218.1	5.9	1.962 ± 0.021	5.9	0.899 ± 0.005	3.0
	132	0.48	16	219.0	9.0	2.134 ± 0.029	8.2	0.976 ± 0.009	3.9
			10‡	213.9	4.7	2.070 ± 0.021	4.8	0.968 ± 0.008	3.7
	133	0.64	16	209.3	13.6	2.106 ± 0.039	11.1	1.010 ± 0.009	5.6
			15†	210.7	13.7	2.101 ± 0.042	11.5	1.000 ± 0.007	4.2
180	13	0.35	14	301.7	4.3	3.112 ± 0.022	3.9	1.032 ± 0.004	2.3
	132	0.48	16	302.4	8.5	3.213 ± 0.037	6.9	1.065 ± 0.007	4.0
	133	0.64	16	303.6	5.8	3.315 ± 0.026	4.8	1.093 ± 0.007	3.7
			14‡	303.5	5.0	3.330 ± 0.021	3.6	1.098 ± 0.007	3.7
1 yr.	13	0.35	18	343.4	7.4	3.756 ± 0.030	5.1	1.096 ± 0.008	4.6
	132	0.48	16	346.2	7.9	3.934 ± 0.036	5.4	1.136 ± 0.008	4.3
	133	0.64	18	346.6	7.6	4.046 ± 0.039	6.1	1.170 ± 0.011	5.9

* C.v. = coefficient of variability.

† One or more animals included in the averages on the preceding line were excluded from this series of revised averages because their apparent calcium content showed marked variation from others in the group.

‡ One or more animals included in the averages on the preceding line were excluded from this revised series in order to obtain a group more representative in body weight of the general population of the same age, sex, and dietary history.

centages of body calcium in males from this superior diet average one-sixth and one-fourth higher, and those in females were one-

third and one-fourth higher, at ages of 2 and 3 months, respectively. This greater gain in *percentage* of body calcium on the moderately enriched and better balanced diet gains added significance from the fact that growth in body weight was more rapid at the same time; so that the superiority in average gain in *amount* of body calcium is even greater than that in percentage. (See also the discussion of correlation coefficients below.)

TABLE II

Calcium Content of Female Rats

Age	Diet No.	Ca in diet	No. of cases	Net weight		Calcium content				
						Total		Per cent of net weight	
				Mean	C.v.*	Mean ± p.e.	C.v.*	Mean ± p.e.	C.v.*
days		*per cent*		*gm.*		*gm.*			
28	13	0.35	26	42.6	11.4	0.319 ± 0.005	11.2	0.750 ± 0.004	4.1
	132	0.48	25	41.6	9.3	0.339 ± 0.004	9.3	0.817 ± 0.007	6.5
			23†	42.0	8.5	0.344 ± 0.004	8.1	0.821 ± 0.005	4.5
	133	0.64	25	40.0	9.6	0.350 ± 0.004	9.7	0.876 ± 0.005	4.6
60	13	0.35	15	113.7	13.2	1.021 ± 0.019	11.0	0.901 ± 0.007	4.5
	132	0.48	15	115.5	9.5	1.131 ± 0.018	8.8	0.980 ± 0.004	2.6
	133	0.64	16	110.8	10.4	1.151 ± 0.019	10.1	1.040 ± 0.006	3.5
90	13	0.35	16	153.0	7.9	1.632 ± 0.024	8.6	1.066 ± 0.005	2.8
	132	0.48	16	155.3	9.9	1.746 ± 0.024	8.1	1.126 ± 0.006	3.3
	133	0.64	16	146.4	9.1	1.738 ± 0.026	9.0	1.187 ± 0.007	3.4
180	13	0.35	6	176.8	5.5	2.189 ± 0.034	5.8	1.238 ± 0.005	1.6
	132	0.48	7	176.0	5.2	2.304 ± 0.018	3.2	1.311 ± 0.011	3.5
	133	0.64	6	184.6	5.8	2.468 ± 0.040	6.0	1.338 ± 0.019	5.1

* C.v. = coefficient of variability.

† Two animals included in the preceding series of averages were excluded from this revised series because their calcium contents showed marked variations from others in the group.

Effect of Increasing the Calcium As Sole Variable in the Nutritionally Better Diet (Comparison of Diets 13, 132, and 133)

From Tables I and II it will be seen that both sexes at all the ages studied showed better gains in percentage of body calcium when the food contained 0.48 per cent of calcium than when it contained 0.35 per cent, and still higher gains on a diet containing 0.64 per cent of calcium in the dry food. Thus *each* of these stepwise increases of about one-third in calcium of the diet results

in increasing the percentage of calcium in the body by about one-tenth to one-twelfth during the period of most rapid growth, and by about one-thirtieth at full maturity.

When examined by the customary statistical procedure, all of the differences in body calcium percentage between animals from Diet 13 (0.35 per cent calcium) and those from Diet 132 (0.48 per cent calcium) are seen to be "statistically significant." Similarly scrutinized statistically, the differences in body calcium percentage resulting from Diet 133 (0.64 per cent calcium) as compared with Diet 132 (0.48 per cent) appear to be "established beyond reasonable doubt" in the cases of males aged 28 and 60 days, and of females aged 28, 60, and 90 days.

Viewing the data in another light, we see that, throughout the period of most rapid growth (1 to 3 months of age in the rat),

TABLE III

Comparison of Rates at Which Animals with Different Dietary Calcium Levels Approach Their Respective Mature Attainments

Diet No.	Per cent Ca in diet	Ratio of per cent of body Ca to that at 1 yr.			
		At 28 days	At 60 days	At 90 days	At 180 days
13	0.35	0.67	0.72	0.82	0.94
132	0.48	0.70	0.76	0.85	0.94
133	0.64	0.73	0.80	0.85	0.94

the animals on the diet with 0.64 per cent of calcium have attained a *percentage* of body calcium which corresponds to that of animals about 50 days older on the diet with 0.35 per cent of calcium.

Furthermore, when the percentages of body calcium at different ages are compared in each case with the percentage attained at full maturity (1 year) *on the same diet,* it appears (as shown numerically in Table III) that the animals receiving Diet 132 with 0.48 per cent calcium are, even on this basis, ahead of those on Diet 13 (which contains 0.35 per cent) during at least the first 3 months of life, or throughout the period of rapid growth; and that animals on Diet 133 (with 0.64 per cent calcium) are, for 2 months at least, or while making at least half of their growth, somewhat nearer their ultimate body composition with respect to calcium than are corresponding animals on Diet 132.

Relation of Body Weight to Percentage of Body Calcium at Certain Ages

In another study (4) it was shown that there is a strong negative correlation between the rate of gain of body weight during a given period and the percentage of calcium in the body at the end of the period.   Investigating, with large numbers of animals all 28 days old, the relation of body weight to body calcification (in animals of the same age and sex, receiving the same diet), and

TABLE IV

Interrelationship between Net Body Weight at a Given Age and Percentage of Body Calcium in Male Rats

Age	Diet No.	No. of cases	Correlation coefficient, r, $\pm$ p.e.
days			
28	13	23	-0.216 ± 0.133
	132	28	-0.205 ± 0.121
	133	26	-0.208 ± 0.125
60	13	15	-0.522 ± 0.125
	132	11	-0.692 ± 0.105
	133	15	-0.667 ± 0.095
90	13	14	-0.252 ± 0.167
	132	10	-0.352 ± 0.185
	133	15	-0.539 ± 0.122
180	13	14	-0.381 ± 0.152
	132	16	-0.572 ± 0.112
	133	14	-0.648 ± 0.103
1 yr.*	13	18	-0.706 ± 0.079
	132	16	-0.714 ± 0.082
	133	18	-0.586 ± 0.105

* Approximately.

applying a somewhat different method of statistical interpretation, Briwa and Sherman (5) observed only a slight tendency for a high body weight to be associated with a low percentage of body calcium (and *vice versa*) in animals of this age.

The present analyses permit a study of this relationship at various ages, from 1 to 12 months, in individuals all within the normal size range for their age.   For this purpose we have computed the correlation coefficient, r, of Pearson, with the results shown in Table IV.   It will be seen that our data, like those of

Briwa and Sherman, show only a low degree of association between degree of calcification and body size at 28 days of age. The data for later ages, however, considered as a whole, point to an important measure of interrelationship between these two factors, greater than average body weight as an individual characteristic tending to be accompanied by lower than average percentage of body calcium, and *vice versa*. Perhaps unexpected are the findings that this interrelationship appears to be at least as marked at the more liberal as at the lower levels of calcium intake, and at least as marked at full maturity as during rapid growth.

SUMMARY

Attainments in the normal developmental increase of body calcium were studied in rats on a better balanced dietary of higher initial calcium content than in previously reported experiments. Increasing the calcium content of this superior diet from 0.35 to 0.48 to 0.64 per cent resulted in successive increases in percentage of body calcium at a given age, the difference induced by each of these stepwise increments being one-tenth to one-twelfth at the period of most rapid growth and about one-thirtieth at full maturity.

The rats on the highest of these levels of intake attained at the age of 2 months a percentage of body calcium which those on the diet with 0.35 per cent calcium required 50 days more to attain.

The statistical significance of the findings and their bearings upon other aspects of growth and development are discussed.

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THE AMPHOTERIC NATURE OF THE PRESSOR PRINCIPLE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND

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The electrochemical nature of the active principles of the posterior lobe of the pituitary gland has never been unequivocally established. It is obvious that a knowledge of their electrochemical properties is exceedingly significant to our concepts regarding the chemical nature of the compounds.

On the basis of an extensive study of the active principles, Kamm and his coworkers (1) concluded that "both active principles are basic bodies, presumably amines." MacArthur (2), on the other hand, stated that the material which he prepared, and which contained both pressor and oxytocic activity, possessed a rather sharp isoelectric point at about pH 5. However, MacArthur did not describe the experimental method used to determine this value, nor did he give the unitage of the material studied.

More recently, Freeman, Gulland, and Randall (3) in the course of investigating methods of purification studied the electrodialysis of the oxytocic principle. The experiments were performed in a three cell apparatus in which the pH of the center cell was maintained constant, the alkalinity of the cathode chamber was controlled by bubbling CO_2 through it, but no attempt was made to control the pH of the anode chamber. With this arrangement, they found that the activity moved into the cathode chamber at pH values up to 8.5; at higher values of pH, the activity did not move at all. They concluded that the oxytocic principle *is a base or is adsorbed on a basic material in the alkaline range.* The

same conclusion was reached from similar evidence concerning the pressor substance. There is, however, another possible interpretation of the results; namely, that the oxytocic and pressor substances are amphoteric with isoelectric points in the alkaline range. On the basis of this interpretation, the failure of the oxytocic and pressor activities to migrate into the anode chamber when the pH of the center cell is as high as 12 would be ascribed to the impossibility of such ampholytes entering a region of such low pH as prevailed in the anode chamber in these experiments. The anode chamber was acid at the beginning of each experiment and was not controlled in any way during the course of the experiment. Although Freeman *et al.* showed that "Savory and Moore's peptone" does migrate into the anode chamber under these conditions, it does not necessarily follow that *all* amphoteric substances could migrate into the anode chamber under these conditions—it would obviously depend on the isoelectric point of the ampholyte.

Das, Ghosh, and Guha (4) have reported results which differed, depending on the experimental method employed. In an apparatus in which membranes were used, the oxytocic activity traveled to the cathode at pH 9.4 and below, and at pH 10 small amounts moved to both the cathode and anode. When no membranes were employed, an isoelectric point was found between pH 5 and 6. No indication was given of the potency of the material used nor of the variation of pH during the experiment.

In the course of studies on the purification of the active principles by electrophoresis which have been conducted in our laboratory, it was observed that they were unable to enter a compartment where the pH was maintained at 12 (5). This behavior indicated that the pressor and oxytocic principles might be amphoteric. Experiments were accordingly designed to establish definitely, if possible, the electrochemical nature of the active principles. The migration of highly purified preparations of the pressor principle in an electric field was studied under conditions such that the pH of the region in which the material was traveling was maintained constant throughout the experiment. The migration of pressor and oxytocic activities was studied over a range of pH values in a three cell apparatus which will be described in detail later, and also in the Tiselius electrophoresis apparatus.

The pressor principle of the posterior lobe of beef pituitary was found to be amphoteric, with an isoelectric point in the region of pH 10.8 in solutions of 0.02 ionic strength. Preliminary experiments with the oxytocic principle indicated that it is also amphoteric and that its isoelectric point is about pH 8.5.

EXPERIMENTAL

Because of the desirability of using small quantities of the highly potent pressor material, an apparatus was constructed which involved the smallest volumes feasible. The apparatus is shown in Fig. 1. The volume of each small section is about 2 cc. and they are separated by sintered glass disks 3 mm. thick which
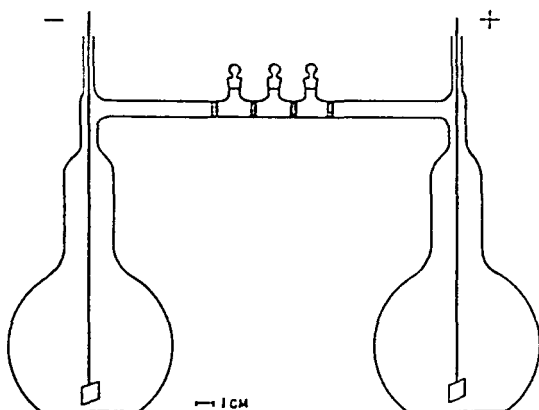
FIG. 1. Horizontal tube apparatus. The volume of each section is 2 cc The sintered glass disks are 3 mm. thick.

had been sealed into the glass. Each electrode vessel contained approximately 600 cc. It was found by measuring the pH of the cells before and after an experiment that this volume of buffer in the electrode vessels was sufficient to keep the pH constant in the region of migration of the material for the duration of a 4 hour experiment. In order to have all the results comparable and to facilitate their interpretation, the salt concentration was always 0.02 M and only uni-univalent buffers were used. Below pH 10, NH_4OH-NH_4Cl buffer was employed; above pH 10, butylamine-butylamine hydrochloride or cyclohexylamine-cyclohexylamine hydrochloride (6) was used. The pH values were determined with the glass electrode.

The pressor material used in these experiments had an activity of 190 units per mg. and was prepared from beef pituitary glands by the method described by Irving, Dyer, and du Vigneaud (5). Unfortunately, the active material could not be dialyzed against the buffer solution, since it passes through membranes. However, at the concentration used, the pH of the buffer was never changed more than 0.05 pH unit by the addition of the pressor material.

The procedure followed in this series of experiments was as follows: The whole apparatus was filled, except the center cell, with the appropriate buffer solution at approximately the same level everywhere. The center cell was then filled with 2 cc. of buffer solution in which 2 mg. of the pressor material had been dissolved. The apparatus was kept in a constant temperature bath at 25°. A thermometer in the center cell indicated that the temperature did not vary appreciably with the passage of current. A potential difference of approximately 115 volts was applied to the system through the platinum electrodes and after a given time (2 to 4 hours) the current was cut off. The contents of each cell were then removed by pipette, were acidified with acetic acid, and were assayed separately for pressor activity. The assays were performed in the usual manner by determining the rise of blood pressure of the dog under chloretone anesthesia.

The results of a series of experiments in which the pH was varied from 9.70 to 11.25 are shown in Table I. Since activity was always found in both the anode and cathode cells, the mobility which is calculated is a net mobility; i. e., the difference in the distance moved in the direction of the anode and the cathode. The net mobility, in $cm.^2$ $volt^{-1}$ $sec.^{-1}$, was calculated from the following relationship,

$$u_{net} = \frac{\text{net fraction moved} \times \text{length of cell (cm.)}}{\text{potential gradient (volt per cm.)} \times \text{time (sec.)}}$$

The potential gradient was determined from the directly measured potential drop from the center of one cell to the center of another. This measured quantity is an average value, since the potential gradient within the disks is considerably higher than in the open tube. However, this is the best approximation that can be made for the calculation of the mobility and at least gives the correct order of magnitude. In Experiment 1, the average potential

gradient was 6.6 volts per cm.; in all other experiments it was approximately 5.4 volts per cm.

In addition to the obvious errors involved in assay and sampling, there are several other errors inherent in this type of apparatus which may affect the quantitative aspects of the results. Owing to the presence of the disks, electroosmosis occurred and there was a mass motion of the solution in the direction of the cathode. However, as soon as a balancing hydrostatic pressure was established, no further motion took place. This phenomenon was actually observed and the volume moved was of the order of 0.1 cc.

TABLE I

Electrophoresis Experiments in Three Section Apparatus at 25°

The beef pressor material had an activity of 190 units per mg.

Experiment No.	pH	Time	Units of activity in each cell			Net fraction moved	Net mobility × 10⁵
			Anode side	Center	Cathode side		
		hrs.					
1	9.70	2	7	207	53	+0.170	+0.844
2	10.20	2	25	192	51	+0.098	+0.580
3	10.25	2	17	277	34	+0.076	+0.455
4	10.40	3	14	280	56	+0.125	+0.495
5	10.55	2	22	330	44	+0.056	+0.343
6	10.55	4	19	273	57	+0.110	+0.325
7	10.90	3	48	275	27	−0.060	−0.237
8	11.25	3	53	150	26	−0.118	−0.466
9*	10.55	4	13	80	33	+0.160	+0.424

* Sample of lower activity, 100 units per mg.

or 5 per cent of the volume of a cell.   On the other hand, the disks were not entirely uniform and, by a preliminary diffusion experiment with an alternating current, it was found that about 4 per cent more of the total material migrated towards the anode chamber than towards the cathode owing to diffusion.   Consequently these two effects, namely the electroosmosis and the asymmetric diffusion, very nearly compensated one another.   However, there always remained the question of some specific effect of the sintered glass disks on the migration which might invalidate the results. For this reason, as well as to obviate the other sources of error already mentioned, it was decided to check the results with the Tiselius apparatus when this technique became available.

The Tiselius apparatus used was the modification described by Longsworth and MacInnes (7). The experiments were run at 0.2° rather than 25° as in the previous series of experiments. The concentration of pressor material was less than 0.02 per cent (2 mg. in 12 cc.). The migration was not observed optically but the contents of the various sections of the cell were analyzed in the usual manner by bioassay. Again the material was not dialyzed, but was dissolved directly in the buffer solution. At the beginning of each experiment, the activity was present only in Sections 2, 3,
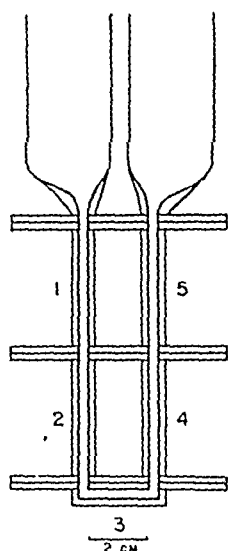
FIG. 2. Cell of the Tiselius electrophoresis apparatus. Section 1 leads to the anode chamber and Section 5 leads to the cathode chamber.

and 4 of the cell as shown in Fig. 2; at the end of the experiment, the amount of pressor activity in all five sections was determined.

The results of a series of experiments with the Tiselius apparatus are given in Table II. In Experiment 1, a borate buffer was used (0.01 M $Na_2B_4O_7$ and 0.02 M H_3BO_3); in Experiments 2 and 3, NH_4OH and NH_4Cl (0.02 M salt); and in Experiments 4 and 5, cyclohexylamine and its hydrochloride (0.02 M salt). Again, the mobility in cm.^{2} volt^{-1} sec.$^{-1}$, given in Table II, is a net mobility, calculated from the relationship,

$$u_{net} = \frac{\text{net fraction moved}}{\text{time (sec.)}} \times \frac{qk}{i} \times \text{length of cell (cm.)}$$

where k is the specific conductance, i, the current, and q, the cross-sectional area; in this cell, q is 0.792 sq. cm. and the length of the

TABLE II

Electrophoresis in Tiselius Apparatus at 0.2°

The beef pressor material had an activity of 190 units per mg.

Experiment No.	pH	Time	Conductance	Current	Units of activity per cc.					Net fraction moved	Net mobility $\times 10^5$
					Anode side, Section 1	Section 2	Section 3	Section 4	Cathode side, Section 5		
		hrs.	ohms^{-1}	milli-amperes							
1	9.25	2	0.791	8.0	1	18	19	19	8	+0.369	+1.886
2	10.25	4.75	1.791	8.2	1.5	28	25	30	3.5	+0.080	+0.300
3	10.70	4	1.593	16.2	7	22	21	22	8	+0.047	+0.115
4	11.25	4	1.095	15.3	15	18	19	9	5	−0.526	−1.11
5	11.64	4	1.097	15.4	22	20		12	8	−0.700	−1.480
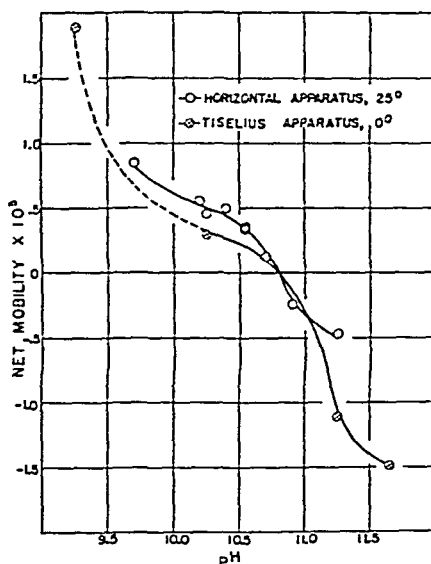
FIG. 3. pH-mobility curves for pressor principle of beef pituitary gland

cell is 4.7 cm. It should be pointed out that the mobility obtained from Experiment 5 (pH 11.64) may be somewhat low, since the material may have traveled beyond the limits of Section 1.

A comparison of the pH-mobility curves of the two series of experiments, namely in the three cell apparatus at 25° and the Tiselius apparatus at 0.2°, is presented in Fig. 3. Both curves show an isoelectric point at about pH 10.85. This point is probably correct within 0.2 pH unit, although the actual values of the mobilities calculated, particularly at 25°, are correct only as far as order of magnitude is concerned. The fact that the active material moves both towards the anode and the cathode at any given pH, in either apparatus, is an effect which is too large to be explained by diffusion alone, and is probably due to differences in the electrical nature of the buffer solution itself and the buffer solution containing dissolved pressor material.

Preliminary investigations of the oxytocic principle yielded evidence that it is also an ampholyte, but its isoelectric point is in the neighborhood of pH 8.5 at 25°.

SUMMARY

A study of the pH-mobility relationship of a highly purified pressor substance of the posterior lobe of beef pituitary glands, carried out in a three cell horizontal electrophoresis apparatus and in the Tiselius apparatus, revealed the amphoteric nature of the pressor substance and the existence of an isoelectric point at about pH 10.85 in buffers of 0.02 ionic strength. A preliminary investigation of the oxytocic principle in highly purified form indicated that it is also amphoteric and has an isoelectric point in the region of pH 8.5.

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BLOOD CHLORIDE AND PHOSPHORUS CONTENT AS AFFECTED BY ADRENALIN INJECTION

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An examination of the literature reveals that investigations regarding the effect of adrenalin on the phosphorus content of the blood are not all in accord. Although most investigators (1, 2) report that adrenalin depresses the inorganic fraction of the blood phosphorus, Yamada (3) finds no significant change, while Seidel (4) and Ichijo (5) record increases. It was observed by Page and Pasternak (6) that the phospholipid content of the blood is reduced following adrenalin injection. Miller (7) does not confirm this in dogs, in which he found inconsistent results in normal animals and a 60 per cent increase when amytal accompanied adrenalin. The effect of this hormone on the chloride content of the blood is likewise uncertain. Tamura (8) and Akiya *et al.* (9) note decreases in the blood chlorides, but Urechia and Retezeanu (10), Chaikelis (11), and Flock *et al.* (12) report no significant variations. In this study we have undertaken to investigate the effect of adrenalin injections on these constituents of the blood of rats, rabbits, and chickens.

EXPERIMENTAL

The adrenalin used was the 1:1000 dilution of adrenalin chloride as prepared by Parke, Davis and Company. Young, healthy, adult male rats of the Wistar Institute strain were employed exclusively. The rabbits were New Zealand white adults weighing from 2 to 3 kilos. Both the rats and the rabbits were fed a balanced ration, with vitamin supplements. The chickens were young Leghorn cockerels and non-laying hens from the college flocks; they were fed the customary growing ration.

After an inanition period of approximately 18 hours, the animals were selected at random and divided into two comparable lots. One group was injected, subcutaneously, with a measured amount of adrenalin solution; massive doses were given in order to obtain the maximum effect and to maintain the blood sugar at a high level for considerable time. Blood was drawn from 60 to 90 minutes after injection by heart puncture, oxalated, pooled, and analyzed. Dilutions for determinations on the whole blood were made at once, and the remainder centrifuged under oil (when chlorides were to be determined) at 3225 R.P.M. for 30 minutes. Specially designed tubes graduated to contain 5 ml. were used, and the cell and plasma volumes were read. The plasma was removed, and the dilution of the cells was made directly in the tube. Since the effect of adrenalin on blood sugar is consistently toward the production of hyperglycemia, blood glucose determinations were made on every sample as a check on the potency and the action of the hormone. The colorimetric method of Benedict (13) was used for the determinations. Chlorides were determined in the whole blood, the plasma, and the cells by the method of Short and Gellis (14); the technique of Wu was followed (15) in preparing the protein-free filtrates. The phosphorus distribution was determined by the method of Youngburg and Youngburg as modified by McCay (16), the colors being read in a photelometer.

DISCUSSION

Adrenalin increases the hexose phosphate content of the muscle. From this evidence it would appear that at least a portion of the inorganic phosphorus reduction in the plasma and, to a lesser degree, in the cells (Table I) might be accounted for by the formation of muscle hexose phosphates. Since adrenalin is known to inhibit the utilization of lactic acid in the body, the increased lactic acid content of the blood and of the muscle might retard the decomposition of hexose phosphates, resulting in an accumulation in the muscle. The reduction in the phospholipid content in the plasma after adrenalin injection is not great in the mammals, the rats and the rabbits, but it is definite in the chickens, in which the concentration of organic phosphorus is much greater. The fact that no significant changes are observed in the organic phos-

TABLE I

Phosphorus Distribution in Blood Following Adrenalin Injection

The phosphorus values are given in milliequivalents per kilo; 1 milliequivalent of phosphorus is calculated as 31 mg.

Animal	No. of animals	Adrenalin injected	Glucose	Cell volume	Total P			Lipoid P		Inorganic P	
					Whole blood	Plasma	Cells	Plasma	Cells	Plasma	Cell
		mg. per kg.	*mM per kg.*	*per cent*							
Rat, control.	39		5.17	50.8	13.60	5.51	24.79	2.86	6.73	2.35*	1.35*
" experimental	34	1.0	17.15	52.1	15.34	4.83	25.08	2.78	6.89	1.96	1.19
Rabbit, control ...	7		5.62	48 2	18 56	5.41	38.59	3.13	7.12	1.32†	1.84†
Rabbit, experimental	7	0.5	17.97	39.7	16.37	3.48	35.07	2.32	7.44	0.58	1.32
Chicken, control.. ...	10		10.82	37.1	39.94	7.22	91.71	5.41*	9.21	2.19*	1.70*
Chicken, experimental	11	1.0	16.80	37.7	41.45	6 93	91 55	3.96	8.73	1.12	1.38

* Of probable statistical significance.

† Highly significant.

TABLE II

Chloride Concentration in Blood Following Adrenalin Injections

Animal	No. of animals	Adrenalin injected	Glucose	Total Cl as NaCl	Plasma Cl	Cell Cl	Cell volume
		mg. per kg.	*mM per kg.*	*m.eq. per kg.*	*m eq. per kg.*	*m.eq. per kg.*	*per cent*
Rat, control..	36		5 77	8.89			51.2
" experimental	39	0 5	13 33	9 48			53.8
Rabbit, control	4		5 70	9 27	11 16	7.58	47.4
" experimental	4	0 5	21 11	9 35	11.04	7.40	46.8
Chicken, control	21		10 39	10 35	11.62	7.24	38.3
" experimental.	23	1.0	16.58	10.30	11.52	7.10	39.8

phorus of the cells is in accord with the findings of Sinclair (17), who believes that the cells are not involved in the transport of phospholipids.

The general stability of the blood chloride level is apparently not markedly affected by adrenalin. The changes indicated (Table II) are not large enough to be very significant; although the glucose increased $2\frac{1}{2}$ to 3 times the normal level, the largest shift in the chloride content of the plasma or of the cells is less than 3 per cent. The changes which were observed indicate that there might be a slight reduction in the chloride content of both cells and plasma.

SUMMARY

The effect of adrenalin injections on the blood phosphorus and chloride distribution has been studied. Data are presented which indicate that in rats, rabbits, and chickens adrenalin reduces the inorganic phosphorus in the plasma and, to a lesser degree, in the cells. A statistical study of these data indicates that this is only probable in the case of the rats and chickens but highly significant in the case of the rabbits. The lipoid phosphorus fraction is reduced markedly in chickens. There are no significant changes in the chlorides in the whole blood, the plasma, or the cells.

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THE ABSORPTION AND FATE OF FREE CITRIC ACID IN THE RAT*

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In marked contrast to the alkalinizing effect and the con-comitant increase in urinary citric acid which is brought about by feeding the alkali salts of citric acid is the almost total disappear-ance of ingested free citric acid and its lack of effect on the reaction of the urine. This behavior of the free acid has been demonstrated in man (Östberg, 1931; Kuyper and Mattill, 1933; Boothby and Adams, 1934; Schuck, 1934), dogs (Sherman, Mendel, and Smith, 1936), rabbits (Langecker, 1934), swine (Fürth, Minnibeck, and Edel, 1934), and rats (Kuether, Meyer, and Smith, 1940). That the metabolism may proceed by way of carbohydrate was indicated many years ago by Greenwald (1914) who observed an increase in urinary sugar after feeding sodium citrate to diabetic patients and to phlorhizinized dogs. Recently, MacKay, Carne, and Wick (1940) published data suggesting that free citric acid shows a glucose-like effect in insulin shock and also that it forms glycogen in the liver in rats. The present study was undertaken to determine the rate of absorption of orally administered free citric acid; observations were also made on the transformation of this compound to hepatic glycogen.

EXPERIMENTAL

Albino rats (Connecticut Agricultural Experiment Station strain) were maintained from weaning on a stock diet consisting

of 16.5 pounds of calf meal,[1] 280 cc. of cod liver oil, 225 gm. of wheat germ, and 225 gm. of dried yeast. For the first 21 days following weaning they were fed, in addition, a paste food consisting of whole milk powder 25, casein 25, wheat germ 20, and lard 30 per cent. In addition each rat received about 10 gm. of fresh lettuce each week. When the rats attained a weight of 130 to 170 gm., all food was removed for 48 hours but the rats were allowed free access to water. At the end of this 48 hour period, one group of fasting animals was killed and the quantity of citric acid in the gut determined. A second group was given by stomach tube 0.9 per cent sodium chloride solution in amounts of 1 cc. per 100 gm. of body weight. The remainder of the animals was given by stomach tube approximately 100 mg. of citric acid per 100 gm. of body weight, in a 10 per cent solution. At the end of $\frac{1}{2}$, 1, 2, 3, and 5 hours, rats of the second and third experimental groups were killed and the citric acid remaining in the gut determined. The amount absorbed is then the difference between the citric acid administered and that recovered minus the material present in the fasting gut.

The rats were killed by a sharp blow on the head. As soon as the reflex struggling had stopped, the abdomen was opened along the linea alba, the thorax being opened at the same time in order to collapse the lungs. The esophagus and rectum were clamped off, the entire gastrointestinal tract removed, stripped of mesentery, and immersed in about 30 cc. of distilled water containing 5 cc. of 50 per cent sulfuric acid. The material was cut into small pieces, brought to boiling, cooled, made up to 100 cc. including 25 cc. of 10 per cent trichloroacetic acid, and filtered after $\frac{1}{2}$ hour. Appropriate aliquots were taken for analysis and citric acid was determined by the method of Pucher, Sherman, and Vickery (1936), the final measurement being made photoelectrically with a color filter having maximum transmission at 4250 Å. In some of the animals the stomach and intestine were analyzed separately; in these the pylorus was clamped off as soon as the abdomen was opened and the stomach and intestine put into separate beakers.

Glycogen estimations were made on the livers of all the animals

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[1] Larro Calf Builder.

according to the method of Good, Kramer, and Somogyi (1933), the livers being removed as soon as the abdomen was opened. The entire operation, from the time the rats were stunned until the livers were placed in the alkali, required no more than 3 minutes. The glycogen was hydrolyzed with 0.6 N hydrochloric acid and glucose determined by the Shaffer-Hartmann method.

The orally administered citric acid which disappears from the gut is either absorbed or is destroyed by the bacteria and enzymes present. To eliminate this second possibility as a pertinent factor, a series of experiments was carried out in which the intestinal contents and intestines of rats were incubated with citric acid under various conditions. In the first experiments, the intestinal contents of four rats (given the same diet as the experimental animals but not fasted) were removed. Two lots were mixed with citric acid and divided into four samples each, two of which were analyzed immediately for citric acid and the other two incubated at 37° for 44 hours, one under aerobic and one under anaerobic conditions. In the other two samples of intestinal contents the pH was measured, a citrate buffer of that pH was added, and these samples then treated the same as were the other two. In both the buffered and unbuffered samples incubated under aerobic conditions the destruction of citric acid was less than 0.5 per cent of the material present, but under anaerobic conditions, the samples which had been buffered to the original pH (6.5) of the intestinal contents showed about 50 per cent destruction, while the samples incubated at the pH of 3.3 existing after the addition of the citric acid showed no destruction.

The stomach and small intestine of the experimental animals fasted for 48 hours were practically empty and since it is generally conceded (Verzár, 1936) that absorption (other than water and salts) normally takes place in the small intestine, two more experiments were carried out under conditions more nearly simulating those existing in the experimental animals. In the first, two fasted rats were given citric acid by stomach tube and immediately killed. The esophagus and rectum were tied, and the entire gastrointestinal tract removed, immersed in Tyrode's solution, and incubated for 5 hours at 37°. In the second experiment the stomach and small intestine of two fasted rats were removed, tied at the esophagus and lower end of the ileum, and, after citric

acid had been injected into the stomach, incubated in Tyrode's solution at 37° for 5 hours. In the animals from which the entire gastrointestinal tract was incubated, there was about 20

TABLE I

Absorption of Citric Acid by Control Groups

Rat No. and sex	Weight	Surface area*	Liver weight	Liver glycogen	Glyco-gen per sq. dm.	Intesti-nal citric acid	Remarks·
	gm.	*sq. dm.*	*gm.*	*per cent*	*mg.*	*mg.*	
27 M.	286					1.18	Fasted only
28 "	301					0.93	
29 "	331					1.22	
30 "	279					1.07	
31 "	251					0.88	
32 "	272					1.43	
43 F.	283	3.92	8.65	0.042	0.93	0.91	
44 "	216	3.28	5.58	0.238	4.05	0.85	
45 "	238	3.49	7.20	0.116	2.40	0.47	
46 "	200	3.11	5.55	0.074	1.31	0.57	
47 "	227	3.39	6.15	0.171	3.11	0.79	
48 "	219	3.31	5.00	0.065	0.98	0.84	
73 "	110	2.09	4.11	0.304	5.98	0.84	
75 "	113	2.13	4.09	0.176	3.38	0.80	
76 "	111	2.10	4.21	0.240	4.81	0.69	
78 "	110	2.09	3.69	0.238	4.20	0.75	
Mean......................				0.166	3.11	0.89	
71 F.	112	2.11	4.67	0.642	14.2	0.83	Given saline and
84 "	108	2.06	4.32	1.042	21.8	0.77	killed after ½, 1,
81 M.	129	2.32	4.96	0.863	18.5	0.80	2, 3, and 5 hrs.
74 F.	119	2.20	4.57	0.950	19.7	0.86	
80 M.	129	2.32	4.46	0.395	7.6	0.75	
· Mean......................				0.778	16.4	0.80	
Grand mean..............................						0.87	

· * Calculated by the formula of Benedict and MacLeod (1928-29).

per cent destruction, but in those from which only the stomach and small intestine were used there was no destruction. All four intestines showed peristaltic movements for about 2½ hours.

In a previous paper (Kuether, Meyer, and Smith, 1940) it was stated that "the intestinal contents of the rat do not destroy citric

acid either at the normal pH of 6.5 or at the pH of 3.3 obtaining after the addition of citric acid." The further findings here reported indicate that under certain conditions, namely at the pH of the intestinal contents and under anaerobic conditions, destruction of citric acid may take place. However, since it has been shown that the stomach and small intestine of the fasted rats do not destroy appreciable amounts of citric acid, it seems safe to assume that under the experimental conditions employed in the absorption studies, the destruction of citric acid in the gut of the rat is negligible. The data on the control animals (Table I) show that under the conditions of prior nutrition and fasting as employed in this study, rats of about 150 gm. of body weight have between 0.8 and 0.9 mg. of citric acid in the gastrointestinal tract and that the reaction of the gut to the introduction of citric acid-free isotonic fluid is not such as to increase its citric acid content.

<center>DISCUSSION</center>

The data on absorption of citric acid are presented in Table II. The absorption coefficient, and hence the rate of absorption, is greatest for the shorter periods, and decreases as the period is lengthened, indicating a decrease in the rate of absorption as the citric acid is removed from the gut. This suggests that the rate of absorption is proportional to the amount of material in the gut.

If, according to our data, a 100 gm. rat were given exactly 100 mg. of citric acid by stomach tube, at the end of the 1st hour it would have absorbed $41.7 \times 1 = 41.7$ mg. of the citric acid present at the beginning of the hour, or 41.7 per cent of it. At the end of the 1st hour there would be 58.3 mg. remaining in the gut. During the first 2 hours it would absorb a total of $34.0 \times 2 = 68.0$ mg., or during the 2nd hour it would absorb $68.0 - 41.7 = 26.3$ mg. This is 45.1 per cent of the citric acid present at the beginning of the 2nd hour, leaving 32.0 mg. unabsorbed. Similarly during the 3rd hour it would absorb 44.4 per cent of the material present at the beginning of the 3rd hour, and during the 4th and 5th hours 38.2 per cent of the citric acid present at the beginning of the 4th hour. The figures relating to the proportion of citric acid present which is absorbed during each hour, namely 41.7, 45.1, 44.4, and 38.2, indicate that the rate of absorption is

TABLE 11
Rate of Absorption of Citric Acid

Rat No. and sex	Weight	Citric acid			Absorption coefficient	Remarks
		Given	Re-cov-ered*	Ab-sorbed		
	gm.	*mg.*	*mg.*	*mg.*	*mg. per 100 gm. per hr.*	
26 F.	121	120	116	4	6.6	Half hr. groups
38 M.	122	120	143	0	0	
42 F.	116	120	99	21	36.2	
55 M.	133	130	117	13	19.5	
56 "	146	150	138	12	16.4	
59 F.	122	120	89	31	50.8	
64 M.	127	130	110	20	31.5	80 mg. recovered in stomach
79 "	110	110	92	18	32.7	68 mg. recovered in stomach
83 F.	116	120	99	21	36.2	72 mg. recovered in stomach
Mean and probable error of mean......................					25.5 ± 3.7	
24 F.	113	120	59	61	54.0	1 hr. group
36 M.	118	120	69	51	43.2	
37 F.	132	130	72	58	43.9	
41 "	112	120	74	46	41.1	
54 "	137	150	95	55	40.2	
58 "	118	120	67	53	44.9	
63 M.	132	130	87	43	32.6	
72 F.	106	110	69	41	38.7	
82 "	110	110	70	40	36.4	
Mean and probable error of mean......................					41.7 ± 1.4	
18 F.	119	120	35	85	35.6	2 hr. group
33 M.	137	135	30	105	38.3	
34 F.	140	140	53	87	31.1	
40 "	124	120	37	83	33.5	
52 "	138	150	45	105	38.0	
57 "	125	120	59	61	24.4	
62 M.	134	130	37	93	34.7	
67 "	122	120	29	91	37.3	
70 F.	118	100	21	79	33.5	
Mean and probable error of mean......................					34.0 ± 1.1	

* Corrected for the amount of citric acid present in the fasting gut.

TABLE II—*Concluded*

Rat No. and sex	Weight	Citric acid			Absorption coefficient	Remarks
		Given	Recovered*	Absorbed		
	gm.	mg.	mg.	mg.	mg. per 100 gm. per hr.	
17 F.	123	120	32	88	23.8	3 hr. group
21 "	126	130	25	105	27.8	
23 M.	120	120	21	99	27.5	
39 F.	112	120	16	104	31.0	
50 "	130	150	30	120	30.8	
53 "	123	120	36	84	22 8	
60 M.	127	130	22	108	28.3	
61 F.	121	120	26	94	25.9	
68 "	100	100	14	86	28.7	
Mean and probable error of mean.. .. .					27.4 ± 0.6	
13 F.	119	120	25	95	15.9	5 hr. group
14 M.	135	135	1	134	19.9	
15 F.	116	120	9	111	19.1	
16 "	125	120	11	109	17.4	
49 M.	226	200	7	193	17.1	
51 F.	121	120	19	101	16.7	
66 "	109	110	3	107	19.6	
69 M.	116	120	12	108	18.6	
77 F.	117	120	14	106	18.1	
Mean and probable error of mean					18.0 ± 0.3	

approximately proportional to the amount of citric acid in the gut at any given time.

On the basis of this reasoning the absorption coefficient for the half hour period should be greater than that for a 1 hour period. However, the value obtained for the half hour period was actually about half that obtained for the 1 hour period. The reason for this appears to be that the administration of the stomach tube causes considerable excitement which inhibits gastric motility and prevents the citric acid from leaving the stomach. This is shown by the fact that in the three animals of the half hour group, in

which the stomach and intestinal contents were analyzed separately, 75 per cent of the citric acid recovered was in the stomach.

It appears, from the present data, that the rate of absorption of citric acid is proportional to the amount of citric acid in the gut. This is in agreement with the findings of Cori and Cori (1929) and of Cori (1930) that lactic acid and sodium lactate are absorbed at a rate dependent on the amount of material present in the intestine. In this respect the organic acids, lactic and citric, differ from the sugars (Cori, 1925) and amino acids (Wilson and Lewis, 1929; Wilson, 1930) which are absorbed at the same rate irrespective of the amount fed and of the duration of the period of absorption.

In the present study, about 90 per cent of the citric acid given in amounts of 100 mg. per 100 gm. of body weight is absorbed within 5 hours.

The data for liver glycogen are presented in Table III; the carbohydrate concentration is referred both to the fresh weight of the liver and to the body surface. Following the administration of citric acid there is an increase in liver glycogen from the half hour period with a peak at the 3 hour period after which the concentration decreases. The rise and subsequent fall can probably be attributed to the fact that there are two opposing tendencies, synthesis and hydrolysis of glycogen, competing with each other after the administration of the citric acid. During the early periods the rate of synthesis is greater than the rate of hydrolysis and the net result is an increase in glycogen. During the later periods the rate of synthesis decreases as a result of the decreased rate of absorption, and, with the continuing hydrolysis, the net result is a decrease in liver glycogen. If these values are plotted graphically, the peak appears to come between the 2nd and 3rd hours.

Since the amount of citric acid that was absorbed by each of these animals is known (Table II), it is possible to estimate the extent to which citric acid is converted into liver glycogen. If the 6 carbon atoms of citric acid are converted quantitatively to glucose and the glucose then dehydrated to glycogen, 192 mg. of citric acid should give rise to 162 mg. of glycogen and the ratio mg. of glycogen per mg. of citric acid would have the value 0.844. MacKay et al. (1940) assumed that Krebs' scheme (1937) for the

oxidation of citric acid by muscle applies to the formation by the liver of glucose from citric acid and deduced that 2 moles of citric acid → 1 mole of glucose. In that case the ratio mg. of glycogen per mg. of citric acid would be just half the above value, or 0.422. They further stated that their "data [on the antiketogenic action of citric acid] suggest that mole for mole citric acid is about half as active as glucose." On the other hand, Greenwald (1914) concluded that citric acid is converted mole for mole into glucose; in five experiments on phlorhizinized dogs the average amount of extra glucose excreted was 90 per cent of that calculated, assuming that the 6 carbon atoms of citric acid are transformed into glucose without loss of carbon.

From Table III it is seen that the liver glycogen value is fairly steady for the 1, 2, and 3 hour groups, indicating that the factors tending to change the values are approximately in equilibrium. These, then, are the logical time intervals at which to calculate the degree of conversion of citric acid to glycogen. The value of the ratio, mg. of extra glycogen per mg. of citric acid absorbed, calculated for these three groups of animals, is found to be 0.7,[2] which approximates the value 0.844 and corroborates the view of Greenwald (1914) that citric acid is transformed mole for mole into glucose. When the value of this ratio is calculated from the data on the glycogenic action of citric acid given by MacKay et al. (1940), the average value for four experiments (thirty-eight rats) is 0.075. For this calculation it is necessary to assume that all the citric acid administered was absorbed, which assumption is probably valid, since it has been shown (Kuether, Meyer, and Smith, 1940) that the absorption exceeds 99 per cent, and further that citric acid is not destroyed in the small intestine of the rat. The reason for the low values obtained by MacKay et al (1940) probably lies in the fact that large amounts of citric acid were fed in several doses over a long period (e.g. Experiment I, 1426 mg. of citric acid fed in nine doses over a period of 64 hours); ample time was thus allowed to metabolize the major portion of the glycogen which may have been formed.

[2] Average of the values for the individual animals in the group; mg. of extra glycogen = weight of liver × (per cent of glycogen minus average per cent of glycogen in control group).

TABLE III
Glycogenic Action of Citric Acid

Rat No.	Weight	Surface area[*]	Liver weight	Liver glycogen	Glycogen per sq. dm.	Group
	gm.	*sq. dm.*	*gm.*	*per cent*	*mg.*	
26	121	2.22	4.69	0.694	14.7	Half hr.
38	122	2.24	5.87	0.775	20.3	
42	116	2.16	4.44	1.059	21.8	
55	133	2.37	4.21	0.473	8.4	
56	146	2.52	4.76	0.191	3.6	
59	122	2.24	4.22	0.270	5.1	
64	127	2.30	4.14	0.568	10.2	
79	110	2.09	4.52	0.657	14.2	
83	116	2.16	4.62	0.279	6.0	
Mean and probable error of mean.........					11.6 ± 1.6	
24	113	2.13	3.99	0.561	10.5	1 hr.
36	118	2.19	4.08	0.818	15.2	
37	132	2.36	5.34	2.035	46.1	
41	112	2.11	4.17	2.343	46.3	
54	137	2.42	4.72	0.468	9.1	
58	118	2.19	4.00	1.132	20.7	
63	132	2.36	4.67	1.017	20.1	
72	106	2.04	3.77	0.480	8.9	
82	110	2.09	4.33	1.044	21.6	
Mean and probable error of mean.........					22.1 ± 3.3	
18	119	2.20	4.68	0.981	20.9	2 hr.
33	137	2.42	6.04	1.424	35.5	
34	140	2.45	5.65	2.670	61.5	
40	124	2.26	5.29	2.024	47.4	
52	138	2.43	4.62	1.141	21.7	
57	125	2.27	4.13	0.349	6.3	
62	134	2.38	4.35	1.104	20.2	
67	122	2.24	4.22	0.974	18.3	
70	118	2.19	3.80	0.455	7.9	
Mean and probable error of mean.........					26.6 ± 4.1	
17	123	2.25	4.80	1.258	26.8	3 hr.
21	126	2.29	4.23	1.160	21.4	
23	120	2.21	4.43	2.009	40.2	
39	112	2.11	4.94	1.992	46.6	
50	130	2.33	4.92	1.248	26.3	
53	123	2.25	4.39	1.050	20.5	
60	127	2.30	4.51	1.133	22.2	
61	121	2.22	5.16	1.653	38.4	
68	100	1.96	4.12	1.764	37.1	
Mean and probable error of mean.........					31.1 ± 2.2	

[*] Calculated by the formula of Benedict and MacLeod (1928–29).

TABLE III—*Concluded*

Rat No.	Weight	Surface area*	Liver weight	Liver glycogen	Glycogen per sq. dm.	Group
	gm.	sq. dm.	gm.	per cent	mg.	
13	119	2.20	4.60	0.935	19.5	5 hr.
14	135	2.39	5.15	1.033	22.2	
15	116	2.16	3.58	1.821	30.2	
16	125	2.27	5.04	1.361	30.2	
49	226	3.38	8.55	1.047	26.5	
51	121	2.22	3.46	0.956	14.9	
66	109	2.03	3.71	0.924	16.5	
69	116	2.16	4.62	0.784	16.8	
77	117	2.18	4.06	0.505	9.4	
Mean and probable error of mean . . .					20.7 ± 1.6	

SUMMARY

Free citric acid administered orally to the albino rat in doses of 100 mg. per 100 gm. of body weight is rapidly absorbed, the rate of absorption being proportional to the amount in the gastrointestinal tract.

The absorbed citric acid disappears as such but appears to be transformed into liver glycogen in a way which suggests a mole for mole relationship.

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VITAMIN K ACTIVITY AND STRUCTURE

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This paper presents the results of assays for antihemorrhagic activity carried out on an array of naphthoquinones and related compounds synthesized as described in a series of previous papers. Some of the compounds were prepared as synthetic models of the structural types postulated (1) for vitamins K_1 and K_2 (1-5); others were synthesized subsequent to the establishment of the structure of vitamin K_1 (6-9) in a study of the relationship of vitamin K activity and structure (10-15). Preliminary bioassay results reported at the outset of the investigations (1, 2) were subsequently recognized as uncertain (3), and judgment was reserved until the assay procedure could be more fully explored. Later reports have included purely preliminary indications of the degree of antihemorrhagic potency encountered in some of the new compounds examined, and the results given in the present paper supersede all previous data.

Assay Method

The assay procedure is a modification of the short curative method described by Ansbacher (16) and is similar to the 18 hour method of Thayer *et al.* (17). Day-old chicks were placed in electrically heated brooders and fed the vitamin K-free diet of Almquist (18-20). Clean water was supplied twice daily and the brooder trays were removed and cleaned daily. After 12 days a trial bleeding was made on ten or twelve birds selected at random from the group. Approximately 0.5 cc. of blood from the brachial vein was collected in a small glass vial which was placed immedi-

ately in the device for rocking at constant temperature described by Almquist and Klose (20). The blood samples were examined at half minute intervals and the time of coagulation determined. When 90 per cent of the birds taken for trial bleeding showed coagulation times of 60 minutes or more, the entire group was considered ready for assay purposes. The birds employed in the preliminary bleeding tests were not used in the assays, since birds so bled frequently have been observed to show a marked reduction in the clotting time within the next 18 hours without any treatment with antihemorrhagic substances. No chicks over 21 days old were employed in the assays.

Except in the case of certain water-soluble compounds, the material to be assayed was dissolved in peanut oil and the solution diluted with the same solvent to such an extent that the quantity of substance to be fed was always contained in exactly 0.1 cc. of the solution. This precaution was taken in order to avoid irregularities in response due to the use of varying volumes of solvent, as observed by Ansbacher (16). After administration of the dose into the crop the birds were held for 18 hours without access to food but with a supply of clean water and the coagulation time of whole blood was then determined. Our criterion for the effective dose is the minimum amount of material which, when administered in 0.1 cc. of peanut oil, will reduce the clotting times of 60 to 80 per cent of the vitamin K-deficient birds to less than 10 minutes in the 18 hour period. Ten or more chicks were used at each dosage level, and with every assay there was included a group of untreated control birds and three groups receiving, respectively, 0.1 γ, 0.3 γ, and 0.5 γ of standard 2-methyl-1,4-naphthoquinone. The latter three groups were included to control possible variation in the degree of deficiency among different lots of chicks. The substance is initially given at a level deemed likely to be ineffective and then at higher levels.

Various units for vitamin K activity have been proposed with dried spinach or alfalfa, extracts of alfalfa, or the more recently suggested 2-methyl-1,4-naphthoquinone (21) or 2-methyl-1,4-naphthohydroquinone diacetate (22, 23) as the reference standard. Pending general acceptance of a reference standard, we have chosen to report our findings in terms of the effective dose in micrograms, but, since 2-methyl-1,4-naphthoquinone has been employed throughout as a comparison control, our results can be

referred to this substance as a standard.   Although some minor variations have been observed, we have found that with the large majority of the groups of deficient chicks investigated 0.3 γ of 2-methyl-1,4-naphthoquinone meets our criterion for the effective dose.

Our most reliable estimate of the minimum dose of pure vitamin K_1 (synthetic) by the above procedure is 1 γ, and hence our results indicate that 2-methyl-1,4-naphthoquinone is 3.3 times as potent as the vitamin in the chick assay.   Almquist and Klose (24) found the ratio 3.8:1, Emmett, Brown, and Kamm (25) found 2.2:1, and Dam, Glavind, and Karrer (23) found 2.1:1.   Ansbacher, Fernholz, and MacPhillamy (26) report a ratio of 4:1 between the potencies of the two compounds when assayed by the 18 hour procedure, but state that the ratio was 30:1 in the 6 hour assay. We have compared the compounds repeatedly by the shorter method without observing any such disparity in the results; by the 6 hour method we find the effective doses of vitamin K_1 and 2-methyl-1,4-naphthoquinone to be 1.5 γ and 0.5 γ, respectively. Thayer *et al.* (21) likewise found no significant difference in the ratio of potencies of the two compounds after assay periods of 6, 18, and 72 hours.   To test the possible influence of the solvent medium, we assayed vitamin K_1 by the 6 and 18 hour methods in cod liver oil, sesame oil, and corn oil, and the results were substantially the same as described for peanut oil.

2,3-Disubstituted Naphthoquinones

The following discussion will be limited to a consideration of the features of structural specificity encountered among substances of pronounced antihemorrhagic activity.   An action so feeble as to be manifested only at a dosage of 1000 γ or more is considered to have little biological significance.   The key point of interest is vitamin K_1, or 2-methyl-3-phytyl-1,4-naphthoquinone (I), and a

(I)

first group of compounds for comparison with this substance embraces those 2-methyl-1,4-naphthoquinones which carry at position 3 some hydrocarbon radical in place of the phytyl group. Table I lists assay results for a series of such compounds and references to papers describing their synthesis and characterization as to purity.

Comparison with results from other laboratories can be made in two cases. Karrer and Epprecht (27) report an assay result

TABLE I

2-Methyl-3-Alkyl- and 3-β-Alkenyl-1,4-Naphthoquinones

Compound	Effective dose
	γ
2-Methyl-3-phytyl-1,4-naphthoquinone (8, 9)	1
2-Methyl-3-farnesyl-1,4-naphthoquinone (11, 14)	5
2-Methyl-3-(β,γ-dihydrophytyl)-1,4-naphthoquinone (11, 15)	8
2-Methyl-3-geranyl-1,4-naphthoquinone (9)	25
2-Methyl-3-cinnamyl-1,4-naphthoquinone (4)	25
2-Methyl-3-(β,γ,γ-trimethylallyl)-1,4-naphthoquinone (4)	50
2,3-Dimethyl-1,4-naphthoquinone	50
2-Methyl-3-benzyl-1,4-naphthoquinone (4)	200
2-Methyl-3-hydrocinnamyl-1,4-naphthoquinone (15)	300

by Dam for the β,γ-dihydride of vitamin K_1 (II) which diverges considerably from ours. Dam found the compound to be only
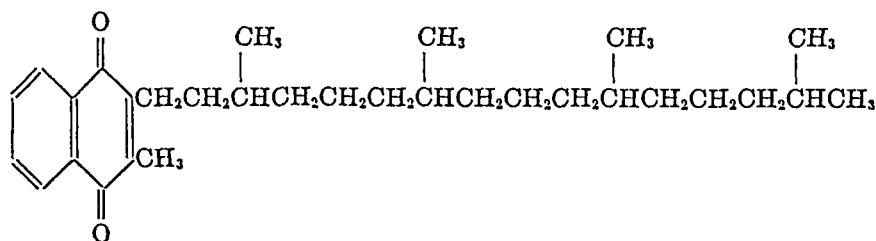
(II)

1/24 as active as the vitamin (23), while we find it to possess 1/8 the potency of the β,γ-unsaturated substance. Karrer and Epprecht prepared the quinone by oxidation of the hydrocarbon and isolated a "practically pure" sample from the reaction mixture by chromatographic adsorption. Our material was prepared by hydrogenation of synthetic vitamin K_1, and contamination with

unreduced starting material is possible but seems unlikely in view of the completely negative color test with alcoholic alkali (15). In the case of this and other liquid quinones we feel a certain confidence in the freedom of our samples from incidental impurities and products of cyclization because of the use of a highly effective method of purification through the solid hydroquinones (8, 9). The effect of saturation of a β,γ double bond has been studied in two other instances and the loss in potency found is of the same order of magnitude as is indicated by our results for the above case. 2-Methyl-3-cinnamyl-1,4-naphthoquinone and its β,γ-dihydride (Table I) constitute a favorable pair for comparison, since they are both crystalline and easily purified, and the latter is found to be 1/12 as potent as the former. In the case of 2-phytyl-1,4-naphthoquinone (Table III), saturation of the double bond of the side chain is attended also with a 12-fold decrease in activity.

Whether the true ratio of the potency of the hydride to that of the vitamin is closer to 1:8 or 1:24, it is clear that the presence of the β,γ double bond is important for maintenance of high potency. The first six quinones in Table I all possess this significant structural feature, and the 2-methyl-3-benzyl compound has the weakly unsaturated phenyl group in the corresponding position of the side chain.

Fernholz, Ansbacher, and MacPhillamy (28) assayed 2,3-dimethyl-1,4-naphthoquinone by their 6 hour method and their results place the compound in much the same position with respect to vitamin K_1 and to methylnaphthoquinone as do ours. Among the compounds listed in Table I the position of 2,3-dimethyl-1,4-naphthoquinone appears rather anomalous, for this simplest member of the series of 2,3-dialkyl compounds possesses neither a long side chain nor a β-unsaturated center but nevertheless exhibits activity of a fairly high degree.

In the series of 3-β-alkenyl compounds the size of the group appears to be a factor of definite significance. The activity bears no proportionality to molecular weight, but decreases in the order of the size of the group, as shown in Table II. The additional double bonds present in the farnesyl and geranyl derivatives evidently are of little influence. It is of interest that the quinone containing the phytyl group (vitamin K_1) would show a still greater superiority in potency over the other compounds if com-

parison were made on a molar rather than on a weight basis. Vitamin K_2 (29), which according to the findings of Doisy and coworkers (30) is probably 2-methyl-3-difarnesyl-1,4-naphthoquinone, is reported to possess from 60 (29) to 67 per cent (23) of the activity of vitamin K_1. Since the molecular weight is 1.3 times that of vitamin K_1, the activity per mole is within about 82 per cent of that of vitamin K_1. In the series of isoprenoid deriva-

TABLE II

Relation of Size of Group to Activity

3-Substituent	Molecular weight of group	Relative activity
CH_3 CH_3 CH_3 | | | $-CH_2CH=CCH_2CH_2CH_2CHCH_2CH_2CH_2CHCH_2C_5H_{11}$ (phytyl)	279	1
CH_3 CH_3 CH_3 | | | $-CH_2CH=CCH_2CH_2CH=CCH_2CH_2CH=CCH_3$ (farnesyl)	205	1/5
CH_3 CH_3 | | $-CH_2CH=CCH_2CH_2CH=CCH_3$ (geranyl)	137	1/25
$-CH_2CH=CHC_6H_5$ (cinnamyl)	127	1/25
$CH_3 CH_3$ | | $-CH_2C=CCH_3$ (trimethylallyl) .	83	1/50

tives the biological activity increases with chain length until a maximum is reached in the 20-carbon residue, and it is not greatly affected by a further elaboration of the chain.

The branched chain isoprenoid structure of the 3-substituent appears to be a factor favorable to the development of antihemorrhagic activity. Thus the straight chain 2-methyl-3-octadecyl-1,4-naphthoquinone (III) has been synthesized by Karrer and Epprecht (27) and by Fernholz, Ansbacher, and MacPhillamy (28) and found by the latter workers and by Dam to be but weakly

active. The quinone is appropriately compared with the β,γ-dihydride of vitamin K_1 (II), and Dam's direct comparison (27) indicates that (III) is less than 1/3 as potent as (II).   The assays
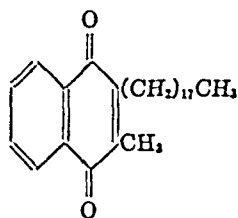
(III)

by Fernholz, Ansbacher, and MacPhillamy of (III) and our assays of (II) would both indicate a still greater disparity between the straight and branched chain compounds.   The octadecyl derivative is a solid melting at about 100°, whereas (II) is a liquid at room temperature, as are the corresponding phytyl, farnesyl, and geranyl derivatives.   Vitamin K_2 is a solid, if a low melting one. It is interesting that biological potency reaches a high point in the liquid, low melting compounds having a branched chain of a certain optimum carbon content, for the situation is reminiscent of that existing among fatty acids having a leprocidal action and those isolated from the tubercle bacillus, as pointed out by Robinson (31).   Another point of interest is that the 2-methyl-3-geranyl and 2-methyl-3-farnesyl compounds exhibit considerable vitamin K potency.   These compounds are easily produced by the condensation reaction applicable to the synthesis of vitamin K_1 (9), and since geraniol and farnesol are widely distributed in nature these lower isoprenologues of vitamins K_1 and K_2 may conceivably occur as additional vitamin K factors.

2-Alkylnaphthoquinones

The observation that 1,4-naphthohydroquinone condenses very smoothly with phytol, farnesol, or geraniol (11, 14) has provided ready access to a series of monosubstituted naphthoquinones of unambiguous structure differing from the compounds of the foregoing section only in the absence of the methyl group.   A substance designated 2-phytyl-1,4-naphthoquinone has been prepared by Karrer and coworkers (32, 33) by another synthesis

and found by Dam (23) to have only 1/240 the activity of vitamin K_1, whereas our material shows 1/50 the potency of the vitamin (Table III). The synthesis of Karrer and coworkers, however, involved establishment of the side chain double bond by a reaction which can proceed in two ways, and these investigators state that

TABLE III

2-Alkyl- and 2-β-Alkenyl-1,4-Naphthoquinones

Compound	Effective dose
	γ
2-Methyl-1,4-naphthoquinone......................	0.3
2-Phytyl-1,4-naphthoquinone (11, 14)................	50
2-Farnesyl-1,4-naphthoquinone (11, 14)..............	500
2-(β,γ-Dihydrophytyl)-1,4-naphthoquinone...........	600
2-Allyl-1,4-naphthoquinone (1, 3).....................	800
2-Geranyl-1,4-naphthoquinone (11, 14)...............	1000 (Slight)
2-Ethyl-1,4-naphthoquinone........................	Inactive at 1000
2-*n*-Propyl-1,4-naphthoquinone (3)....................	" " 1000

the material obtained probably was not completely homogeneous but contained the γ,δ-unsaturated isomer. Evidence cited above
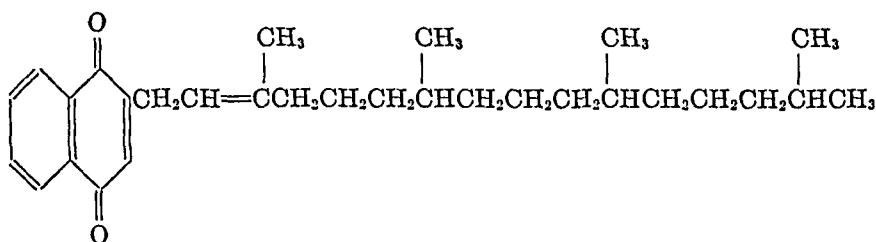
(IV)

shows that any such isomerism should detract from the activity of the sample. A mixture of bond isomers would be expected to show a potency somewhere between that of pure (IV) and its β,γ-dihydride, or from 1/600 to 1/50 the activity of vitamin K_1. As a check on our results for (IV) three independent samples were prepared, one at Harvard and two at the Merck laboratories, and assayed at different times; they all evoked essentially the same biological response.

The monosubstituted naphthoquinones having isoprenoid side

chains exhibit the same relationships encountered in the 2-methyl-3-alkyl series. The activity again decreases in the order phytyl > farnesyl > dihydrophtyl > geranyl, and these compounds are 1/50, 1/100, 1/75, and 1/40 as active as the corresponding methylated quinones. The liquid dihydrophytyl compound appears to be somewhat more active than the crystalline 2-n-hexadecyl- and 2-n-octadecyl-1,4-naphthoquinones synthesized and assayed by Fernholz, Ansbacher, and MacPhillamy (28).

Our early report that 2-ethyl- and 2-n-propyl-1,4-naphthoquinones are decidedly less effective than the methyl homologue (34) has been confirmed by others (28, 35), and our present assays indicate that neither compound possesses activity of any biological significance. The 2-allyl compound appears from present indications to be effective at 800 γ.

2-Methyl-1,4-naphthoquinone clearly occupies a unique position, for it is some 170 times as potent as the next most active of the monosubstituted naphthoquinones (phytyl) and about 3.1 times (average) as active as vitamin K_1. It has been suggested by one of us (9) that the remarkable potency of methylnaphthoquinone may be due not to the functioning of the compound as such but to its conversion in the organism into a quinone of the true vitamin K type. The simple quinone in the reduced form condenses readily under mild conditions with the naturally occurring phytol, farnesol, and geraniol, and presumably would react with other β-unsaturated isoprenoid alcohols to give antihemorrhagic products. A rapid biosynthesis is thus entirely conceivable. Material given by mouth may combine in the gut with one of the reactive alcohols derived from foodstuffs, while that administered parenterally may be supplied with an isoprenoid side chain by a process of synthesis in the liver. In the latter case the alcoholic component may be vitamin A.

If some such transformation of administered methylnaphthoquinone does occur, a given weight of the simple quinone could give rise to much more than its own weight of a vitamin K type of compound. The ratios of molecular weights are such that 1 part by weight of methylnaphthoquinone is equivalent to 2.6 parts of vitamin K_1 or 3.4 parts of vitamin K_2. The assays of Dam *et al.* (23), which provide a direct comparison of all three compounds, indicate that a given weight of methylnaphthoquinone

possesses antihemorrhagic activity equivalent to that of 2.1 parts of vitamin K_1 or of 3.1 parts of vitamin K_2. A quantitative biological conversion to either vitamin thus would more than account for all of the activity observed. While some of the other estimates of the relative potencies of methylnaphthoquinone and vitamin K_1 give a picture which is not quite so favorable to the hypothesis, the average ratio of 3.1:1 is not widely different from the inverse ratio of the molecular weights. It may be argued that a condensation reaction linking phytol to methylnaphtho-hydroquinone would have to proceed more efficiently in the organism than has been realized in the laboratory in order to account for the relationship, for the maximum yield in the vitamin K_1 synthesis is only about 30 per cent (9). A superior efficiency of the biological process seems entirely possible, and furthermore it must be noted that the actual alcoholic component may well be a substance more reactive than phytol and may possibly give a vitamin K principle more potent than the quinones isolated from alfalfa or fish meal. The substance also might differ somewhat from these quinones in therapeutic qualities and persistence.

The approximate equivalence of vitamin K_1 and methylnaphtho-quinone on a molecular basis would also be consistent with the hypothesis of a transformation of the former substance into the latter in the body, but an experimental basis for such a postulate is lacking. There are no indications that the synthesis can be reversed, and degradative cleavage of vitamin K_1 under mild conditions affords phthiocol and not methylnaphthoquinone (8, 9). Furthermore, the 3-phytyl, 3-farnesyl, 3-geranyl, and 3-cinnamyl derivatives of 2-methyl-1,4-naphthoquinone differ in potency much more than would be expected if they function as precursors of a common product of biodegradation. The alternate hypothesis of a biosynthesis is thus more plausible. The case is in no wise weakened by the fact that 1,4-naphthoquinone is very feebly active and falls far short of being equivalent in potency to 2-phytyl-1,4-naphthoquinone. 1,4-Naphthoquinone differs significantly from its 2-methyl derivative in being highly susceptible to addition reactions, and the bulk of the quinone administered may well be diverted from the condensation with an isoprenoid alcohol by interaction with an amino acid or a protein. The 2-methyl group should protect the material, while in the oxidized form, from such dissipating side reactions.

In view of the many indications of a specificity associated with the 2-methyl-3-(β-alkenyl)-1,4-naphthoquinone structure, the apparent glaring exception of the 2-methyl compound is in itself an argument in favor of the above hypothesis. In the monoalkyl series antihemorrhagic activity is observed over a considerable range of chain length, and yet the high potency of the 2-methyl compound is completely abolished on lengthening the chain by a single carbon atom. In terms of the hypothesis of a conversion in the body, the lack of activity of ethylnaphthoquinone finds a simple explanation on the basis of the observation that 2-ethyl-3-phytyl-1,4-naphthoquinone is practically devoid of activity (Table IV).

TABLE IV

Highly Alkylated Naphthoquinones

Compound	Effective dose
	γ
2-Ethyl-3-phytyl-1,4-naphthoquinone (8, 9)	1000
2,3-Diallyl-1,4-naphthoquinone (2, 3)	1000
1,1-Dimethyl-3-*tert.*-butyl-1,4-dihydroanthraquinone (5)	Inactive at 1000
2-(δ-Methyl-γ-pentenyl)-1,4-dihydroanthraquinone (5)	" " 1000
2,6-Dimethyl-3-phytyl-1,4-naphthoquinone (9)	" " 1000
2,5-Dimethyl-1,4-naphthoquinone (15)	500 (Slight)
2,6-Dimethyl-1,4-naphthoquinone	Inactive at 1000
2,7-Dimethyl-1,4-naphthoquinone	1000
2,8-Dimethyl-1,4-naphthoquinone (15) .	500 (Slight)
6,7-Dimethyl-1,4-naphthoquinone (2, 3)	Inactive at 1000

Extension of the 2-Substituent and Alkylation in Benzenoid Ring

The quinones included in Table IV are for the most part monomethyl homologues of vitamin K_1 or 2-methyl-1,4-naphthoquinone, and the lack of any very significant activity in the series as a whole shows that this slight modification in structure results in the almost complete loss of the potencies characteristic of the parent substances. Replacement of the 2-methyl group of vitamin K_1 by a 2-ethyl group gives a compound (V) which possesses only 1/1000 the activity of the vitamin. Inasmuch as the chain of the 3-substituent group can be lengthened or shortened over a considerable range with relatively minor variation in activity, it is remarkable that the least possible extension of the 2-substituent
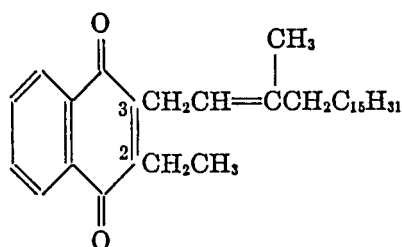
(V)

group, which involves increasing the molecular weight by only 3 per cent, should lead to almost complete abolishment of vitamin K activity. Another comparison of interest can be made between 2-methyl-3-geranyl-1,4-naphthoquinone ((VI), see Table I) and 2-(δ-methyl-γ-pentenyl)-1,4-dihydroanthraquinone (VII). The latter compound is closely related to (VI) and differs only in
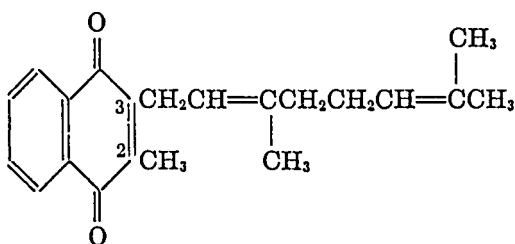
(VI)
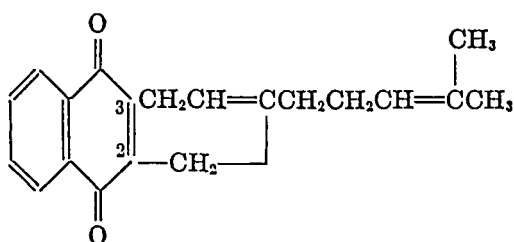
(VII)

having a methylene bridge extending between the 2 position and the γ-carbon atom of the side chain in place of methyl groups at both points. In contrast to (VI), which is effective at a level of 25 γ, (VII) is completely inactive at 1000 γ. For highest potency, the 2-methyl group evidently must be intact, and a lengthening of the group or its incorporation in a side ring interferes very seriously with effective physiological functioning of the quinone.

Karrer and Epprecht (27) recognized this structural relationship and its implications regarding the structure of vitamin K_2.

The specificity with regard to the 2 position is striking, but so is that with respect to substitution in the benzenoid ring. Thus 2,6-dimethyl-3-phytyl-1,4-naphthoquinone (VIII) is almost wholly devoid of activity, showing that a methyl group at the 6

(VIII)   (IX)
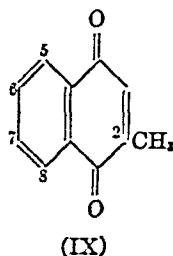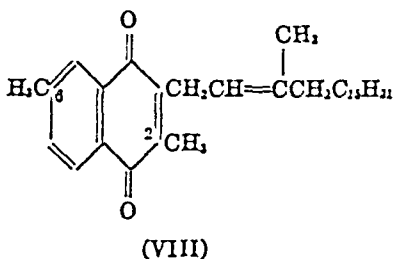
position has the same inhibiting influence as one substituted into the 2-methyl group. Of the five possible methyl derivatives of the potent 2-methyl-1,4-naphthoquinone, only 2,3-dimethyl-1,4-naphthoquinone (Table I) shows appreciable activity (about 1/170 that of the 2-methyl compound). Substitution into the structure (IX) of a methyl group at any one of the positions 5 to 8 results in almost complete deactivation. Since the specificity with regard to the absence of a methyl group from one part of the molecule is comparable with that concerning its presence at another position, there seems to be little foundation for supposing that the favorably located 2-methyl group is involved in the functioning of the antihemorrhagic agent.

Naphthoquinones with Oxygen-Containing Substituents

Phthiocol (X) was the first simple compound reported to have antihemorrhagic activity (36), and both this substance and lawsone have been assayed in several laboratories. The results are in substantial agreement in indicating that the former substance is rather weakly active and the latter either inactive (Table V) or feebly active (23). Phthiocol is an end-product of the color reaction of vitamin K_1 with alcoholic alkali (8, 9) and can be converted in low yield into vitamin K_1 by condensation in the reduced form with phytol (13). While the latter observation suggests the possibility that administered phthiocol may owe its

(X)   (XI)
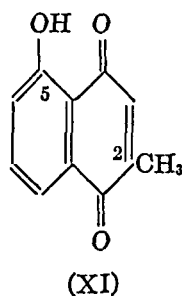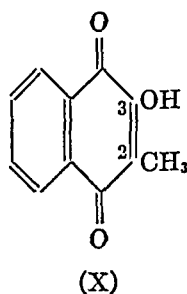
activity to a biological conversion to a vitamin K type principle, the fact that the isomeric plumbagin (XI) exhibits activity of

TABLE V

Hydroxy- and Carbethoxynaphthoquinones

Compound	Effective dose
	γ
Plumbagin, or 2-methyl-5-hydroxy-1,4-naphthoqui-none (37)	400
Phthiocol,* or 2-methyl-3-hydroxy-1,4-naphthoquinone	500
Juglone, or 5-hydroxy-1,4-naphthoquinone	Inactive at 1000
Lawsone, or 2-hydroxy-1,4-naphthoquinone	" " 1000
Lapachol*	" " 1000
2-β-Heptenyl-3-hydroxy-1,4-naphthoquinone*	" " 1000
Crude 2-farnesyl-3-hydroxy-1,4-naphthoquinone (15)	" " 1000
2-Methyl-3-(γ-hydroxydihydrophytyl)-1,4-naphtho-quinone (13)	" " 1000
Hydroquinone diacetate (13)	" " 1000
2-Methyl-3-carbethoxy-1,4-naphthohydroquinone† (38)	25

   * From the collection of Samuel C. Hooker.
   † We are indebted to Dr. C. F. Koelsch for this sample.

about the same order points more in the direction of a weak effectiveness of both compounds acting as such. Thus the introduction of a hydroxyl group into 2-methyl-1,4-naphthoquinone at either the 3 or 5 position results in a profound reduction of antihemorrhagic activity without its complete abolishment. A deactivating influence attending hydroxyl substitution is clearly evident in the results for farnesylhydroxynaphthoquinone and for the 2-methyl-3-γ-hydroxydihydrophytyl compound (XII). The lack of activity of (XII) is striking, for this is the γ-hydroxy
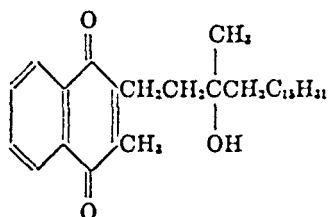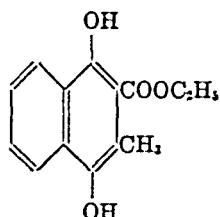
(XII) (XIII)

derivative of β,γ-dihydrovitamin K_1, which is fully effective at a dosage of S γ. The hydroxyl group in this case is alcoholic, whereas in the other instances cited it is phenolic. It is interesting to note that in the field of the similarly lipid-soluble carcinogenic

TABLE VI

Naphthoquinone Oxides

Compound	Effective dose
	γ
Vitamin K_1 oxide (11, 15)	1.2
2-Methyl-1,4-naphthoquinone oxide (4)	5
2,3-Dimethyl-1,4-naphthoquinone oxide (11, 15) .	25
2-Methyl-3-cinnamyl-1,4-naphthoquinone oxide (11, 15)	150
2-Phytyl-1,4-naphthoquinone oxide (11, 15)	200
2-Farnesyl-1,4-naphthoquinone oxide (11, 15)	1000
2,7-Dimethyl-1,4-naphthoquinone oxide (4)	Inactive at 1000

hydrocarbons the introduction of both alcoholic and phenolic hydroxyl groups at various positions results in a complete or considerable loss of biological activity (39).

The last compound listed in Table V is a hydroquinone ester of the structure (XIII), and the high degree of activity (25 γ) is noteworthy. The substance may function in the form of the corresponding quinone, which would then be in a class with 2,3-dimethyl-1,4-naphthoquinone, a compound effective at a not greatly different dosage level (50 γ). Another possibility is suggested by the observation of Koelsch and Byers (38) that esters of this type can be converted very readily into the 2-alkyl-1,4-naphthoquinones by saponification and oxidation. A partial conversion of (XIII) in the organism to methylnaphthoquinone would account for the observed activity.

Naphthoquinone Oxides

The colorless oxides listed in Table VI are easily prepared from the yellow quinones in almost quantitative yield (4), and they have been found capable of reduction under very mild conditions (11, 15) with elimination of the oxidic bridge and formation of the corresponding hydroquinone. This generally applicable reduction reaction affords a basis for the hypothesis that the antihemorrhagic

$$\text{Vitamin K}_1 \xrightarrow{\text{NaOOH}} \text{(XIV)} \xrightarrow{\text{Na}_2\text{S}_2\text{O}_4}$$

with structure (XIV) bearing $CH_2CH{=}CCH_2C_{16}H_{31}$ and CH_3 groups

(XIV)

Vitamin K$_1$ hydroquinone
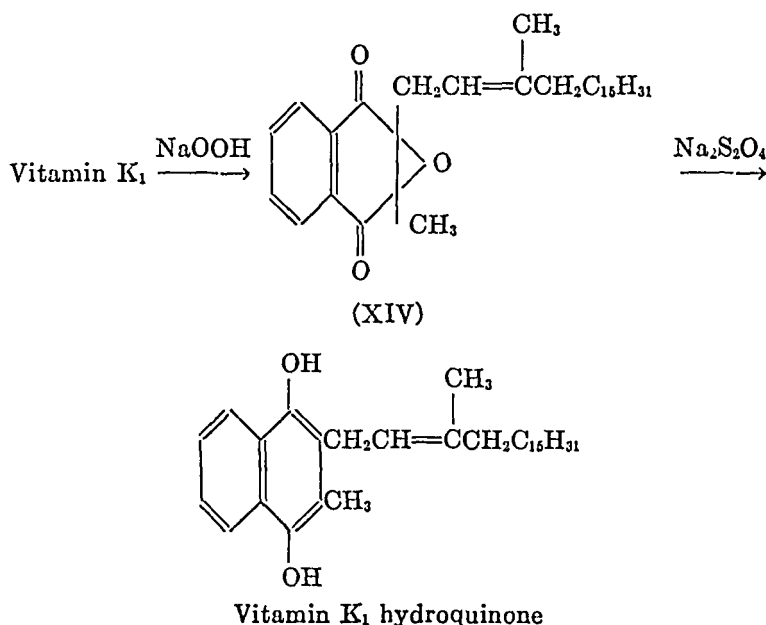
activity observed with certain members of the series may be due to a similar reduction of the administered material in the organism, with formation of either the quinone or the biologically equivalent hydroquinone component of the oxidation-reduction system. This would account for the very high potency of vitamin K$_1$ oxide (XIV), which is hardly distinguishable in the bioassays from the vitamin itself. That 2-methyl-1,4-naphthoquinone oxide (XV) is considerably less potent than (XIV) and exhibits only 1/17 the activity of methylnaphthoquinone may be an indication that the reversion of this oxide to the quinone proceeds less efficiently than in the case of the vitamin oxide. Such an inference is supported by the observation that the oxides of monoalkylated quinones can enter into a second type of chemical transformation

to which the dialkyl compounds are not amenable. The oxide of the 2-methyl compound can be converted smoothly with sulfuric acid at a low temperature into phthiocol (40). Under the influence of alkali it is in part isomerized to phthiocol and in part degraded to lawsone, with loss of the methyl group (15). A partial transformation of administered oxide to either compound

(XV) → Phthiocol
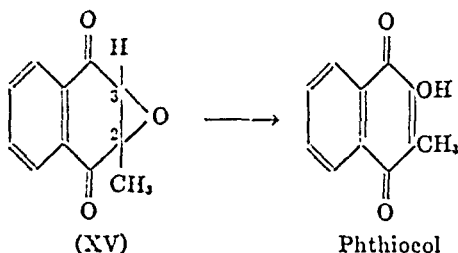
would involve a distinct loss in total potency, and the former change, since it requires merely the migration of the hydrogen atoms at position 3, may well occur to some extent in the course of the test. A 2,3-dialkylnaphthoquinone oxide such as that of vitamin K_1 (XIV) has no hydrogen available for isomerization. Of the other two oxides of the same type which were examined (Table VI), the 2-methyl-3-cinnamyl derivative retains about 1/3 of the activity of the parent quinone, while the oxide of 2,3-dimethyl-1,4-naphthoquinone was found to possess about twice the potency of this compound. The latter result appears anomalous and inconsistent with the hypothesis that the oxides function merely as precursors of the quinones from which they are derived. Perhaps also inconsistent is the fact that the oxides of 2-phytyl- and 2-farnesyl-1,4-naphthoquinones show 1/4 to 1/2 the potencies of the quinones, although the difference from the behavior noted in the 2-methyl series may well be due to a hindering influence of the large isoprenoid groups manifested in protecting the oxide linkage from hydrolytic cleavage. It may also be that differences in absorbability of the oxide and the quinone account for variations in relative potency of the order of from 1:4 to 2:1, noted with all oxides studied except that of methylnaphthoquinone. The pronounced deviation of this compound from the general rule seems attributable to a greater chemical reactivity, with consequent greater opportunity for a diversion of a part of

the material to less potent by-products, and this interpretation holds good whether the oxides function as such or by virtue of a reduction to the quinones.

The highly potent vitamin K_1 oxide may find practical application by virtue of its stability, the substance being much less sensitive to light than the vitamin. Thus solutions of the two substances in petroleum ether (20 mg. in 25 cc.) were exposed together to direct sunlight for 2 hours and then assayed. The vitamin sample had lost about 90 per cent of its activity in this period, while the oxide showed a diminution in potency of less than 30 per cent. This comparatively light-stable derivative, which is easily produced from and converted into vitamin K_1, may possibly have a bearing on the question of the form in which the light-sensitive vitamin exists in green plants. Indeed, Fernholz, Ansbacher, and coworkers (26, 41) report the isolation from alfalfa concentrates of a colorless fraction having properties reminiscent of those of our oxide. Thus their most potent concentrates, like the oxide, gave no purple-blue color with alcoholic alkali and showed about the same activity as vitamin K_1 in the 18 hour assay.

Compounds of Other Than the Naphthoquinone Type

A number of quinones other than those of the α-naphthoquinone series have been investigated in various laboratories, but where antihemorrhagic activity has been encountered it has been of a low order. The only such exploratory assays included in the present report are those for the first few compounds listed in Table VII. The first is analogous in structure to 2-methyl-1,4-naphthoquinone, the second is a benzoquinonoid model of the active 2,3-dimethyl compound, and the third, of formula (XVI),
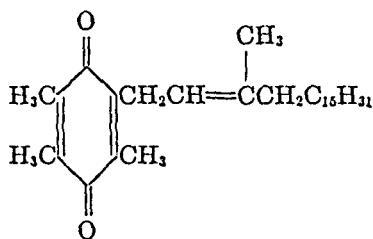
(XVI)
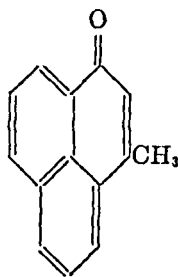
(XVII)

differs from vitamin K_1 only in having two methyl groups in place of the 4-carbon benzenoid side ring. The absence of activity in all three compounds is a further indication of the specificity associated with the structures of the corresponding naphthoquinones. 9-Methylperinaphthenone-7 (XVII) resembles 2-methyl-1,4-naphthoquinone in having an α,β-unsaturated ketonic system with a methyl group at the β position. The lack of activity suggests that the character of the unsaturated system is of importance only if this is present in a compound of the true quinone type.

The naphthotocopherol (XVIII) assayed in the present investigation was prepared by a method which precludes its contamination with the isomeric ketonic substance (XIX), which is formed

TABLE VII

Miscellaneous Compounds

Compound	Effective dose
	γ
2,3,5-Trimethyl-1,4-benzoquinone	Inactive at 1000
Duroquinone	" " 1000
2,3,5-Trimethyl-6-phytyl-1,4-benzoquinone (11, 14)	" " 1000
α-Tocopherylquinone (14)	" " 1000
9-Methylperinaphthenone-7 (15)	" " 1000
Naphthotocopherol (11, 13)	500
2-Methyl-2-phytyl-2,3-dihydro-1,4-naphthoquinone (11, 13)	50

in considerable amounts as a by-product in the synthesis of vitamin K_1 and which, like (XVIII), is not extracted by Claisen's

(XVIII)   (XIX)
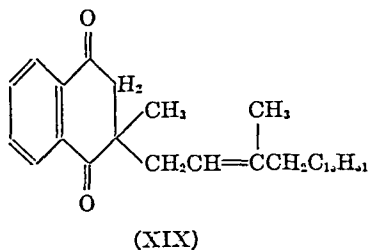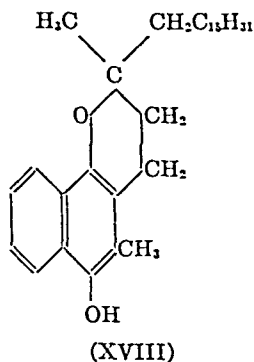
alkali. The naphthotocopherol occupies a unique position in that it evokes the biological responses characteristic of both vitamins E and K (13). The manifestation of antihemorrhagic activity of even a rather low order (500 γ) raises an interesting point. The substance conceivably is convertible into a quinone by either oxidation or reduction. Chemical oxidation gives the naphthotocopherylquinone, or γ-hydroxy-β,γ-dihydrovitamin K_1, but this substance is inactive (Table V). A partial, inefficient reductive cleavage of the chroman ring would explain the observed activity, for the β,γ-dihydride of vitamin K_1 which would result is effective at 8 γ.

That the ketonic by-product (XIX) shows activity at 50 γ seems remarkable. To be sure, the substance is isomeric with vitamin K_1 hydroquinone and it has been found convertible into the vitamin in low yield by a process of pyrolysis and oxidation (11, 13). A biological conversion proceeding to the extent of 1 to 2 per cent would thus appear at least possible.

Naphthohydroquinone Esters and Ethers

Inorganic Esters—Among water-soluble antihemorrhagic compounds of various types which have been suggested for use in parenteral therapy, a series of phosphate and sulfate esters of types (XX) and (XXI) were prepared and examined in our

(XX)   (XXI)
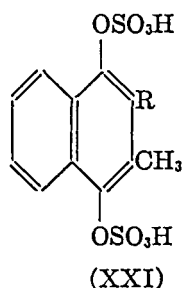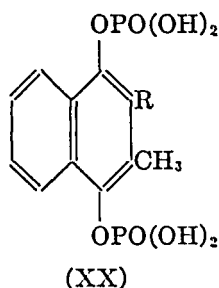
R = H or phytyl

laboratories (10). Subsequently Foster, Lee, and Solmssen (42) published the results of a careful pharmacological study of the diphosphate ester of methylnaphthohydroquinone, and Ansbacher, Fernholz, and Dolliver (43) reported assays for this compound and for the corresponding sulfate ester. Our completed assays

(Table VIII) agree very closely with those of Foster, Lee, and
Solmssen and are at variance with those of the second group of
workers. We find the diphosphate ester to be effective at a dosage
of 0.5 γ, an amount which is equivalent to only 0.2 γ of methyl-
naphthoquinone, and, since our value for the minimum dose of
the latter compound is 0.3 γ, it is evident that on a molecular
basis the ester is at least as potent as the quinone. We agree with
Foster, Lee, and Solmssen on this point, but discount their sug-
gestion that the action of methylnaphthoquinone or a similar
compound is mediated through a phosphate ester, for the cor-
responding ester derived from vitamin K_1 should in this case show

TABLE VIII

Water-Soluble Inorganic Esters

Compound	Effective dose	
	Oral	Intra-ve-nous
	γ	γ
Sodium 2-methyl-1,4-naphthohydroquinone di-phosphate (10).........	0.5	0.5
Sodium 2-methyl-1,4-naphthohydroquinone disul-fate (10).....................	2	1–2
Vitamin K_1 hydroquinone diphosphoric acid (10).	50	10
Sodium 2,3-dimethyl-1,4-naphthohydroquinone disulfate (10)...	500	
Potassium vitamin K_1 hydroquinone disulfate (10).	Inactive at 500	

much higher potency than is actually observed (Table VIII).
This derivative of the vitamin is about 5 times as potent when
given intravenously as when administered by mouth, whereas no
appreciable differences were observed when the diphosphate and
disulfate esters of the 2-methyl compound were assayed by these
two methods. Another point of difference is that the hydroqui-
none esters of vitamin K_1 are much less active than the parent
quinone, whereas the esters of methylnaphthoquinone exhibit
molecular potency of the same order of magnitude as this sub-
stance.

We consider the most likely interpretation of the results to be
that the inorganic esters undergo more or less complete hydrolysis

in the body with liberation of quinones in the reduced form and that the latter are the agents which actually function. The differences noted would then signify that the phosphates are more easily hydrolyzed than the sulfates, which is compatible with biochemical information, and that a given ester of 2-methyl-1,4-naphthohydroquinone is more readily hydrolyzed than the ester of 2-methyl-3-phytyl-1,4-naphthohydroquinone. The latter proposition is reasonable, for in the second case both ester groups are subject to the hindering influence of an adjacent alkyl group, and one of these is a bulky isoprenoid side chain.

Organic Esters and Ethers—Interest in acylated hydroquinone derivatives of quinones possessing antihemorrhagic activity originated in the observation by Doisy and coworkers (44) that the

<div align="center">

TABLE IX

Organic Esters and Ethers

</div>

2-Methyl-1,4-naphthohydroquinone	Effective dose
	γ
Diacetate (4)	1
Dibenzoate (4)	1
Dimesitoate (12, 15)	300
Monoethyl ether (13)	1
Dimethyl ether	5
Dibenzyl " (4)	7

crystalline hydroquinone diacetates of vitamins K_1 and K_2 are about 1/2 as active as the quinones. The diacetate of 2-methyl-1,4-naphthohydroquinone has been assayed in several laboratories and the corresponding dibenzoate and dimethyl ether have been studied by Ansbacher, Fernholz, and Dolliver (45). Our results for these esters and ethers (Table IX) are in substantial agreement with those of other investigators.

As a test of the obvious hypothesis that the acylated naphthohydroquinones undergo hydrolysis in the organism, and exert an antihemorrhagic action merely by virtue of the functioning of the liberated hydroquinone, methylnaphthohydroquinone dibenzoate was compared with the highly hindered dimesitoate (XXII). The latter ester is very resistant to hydrolysis, and the results indicate that it possesses no more than about 1/300 the potency of the

dibenzoate.   This observation lends plausibility to the hypothesis
mentioned.

(XXII)
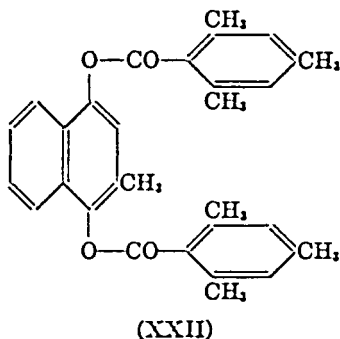
The ethers of methylnaphthohydroquinone would be expected
to be much more resistant to hydrolysis than the acetates and, on
observing that the dimethyl ether is nevertheless about 1/10 as
active as the quinone, Ansbacher, Fernholz, and Dolliver (45)
were inclined to favor the idea that the hydroquinone derivatives
in general function as such and not in the form of the hydroqui-
none-quinone system.   Our assays place the dimethyl ether in
the same relative position with regard to activity.   The dibenzyl
ether, which probably is subject to more ready hydrolytic fission,
appears somewhat less active on a weight basis but actually is
slightly more potent than the dimethyl ether on a molecular basis.
The monoethyl ether of the probable structure (XXIII) is signifi-
cantly more active than either of the diethers, and it occurred
to us that this ether, being of a type susceptible to oxidation, might
be convertible to the quinone in the organism by an oxidative
mechanism.   The observation of Doisy and coworkers (46) that

(XXIII)
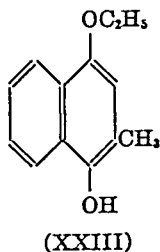
1,4-diacetoxy-2-methylnaphthalene-3-acetic acid is very resistant to hydrolytic fission but can be converted to the corresponding quinone by oxidation suggested that a diether might also be susceptible to oxidation. In a trial experiment it was found that the methylnaphthohydroquinone dimethyl ether (XXIV) can indeed be converted smoothly into the corresponding quinone (XXV) by oxidation with chromic acid (12). It therefore seems

$$\xrightarrow[\;60°\;]{CrO_3}$$

(XXIV)   (XXV)
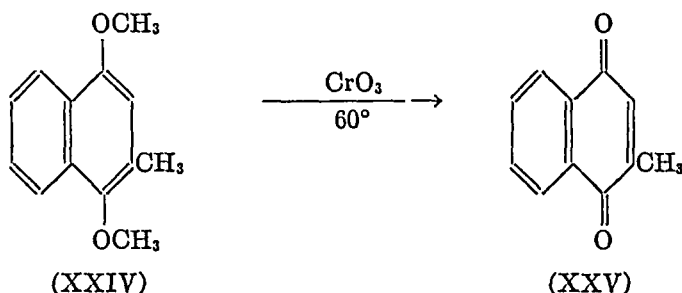
possible that hydroquinone esters and ethers function as antihemorrhagic agents by virtue of a biological conversion to the hydroquinones or quinones by either the hydrolytic or the oxidative route.

Hydrogenated Naphthoquinones

An interesting field for the further consideration of the possible biological transformation of the administered material is provided by a series of hydrides (Table X) obtained in part by catalytic hydrogenation of vitamin K_1 or methylnaphthoquinone, and in part by an independent method of synthesis. The 5,8-dihydride

(XXVI)   (XXVII)
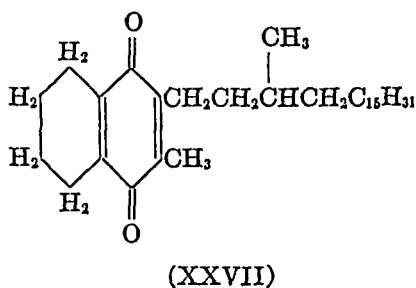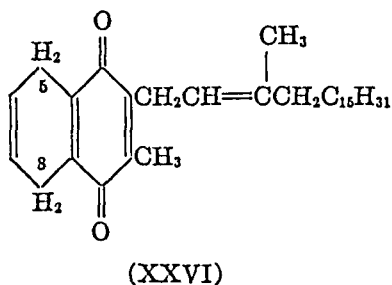
of vitamin K_1 (XXVI) is 1/4 as active as the vitamin and twice as potent as the isomeric β,γ-dihydride (Table I). Partially hydrogenated quinones of this type are easily converted into the fully

aromatized naphthoquinones by chemical oxidation, the hydrogen atoms at positions 5 and 8 being highly activated and subject to either direct attack or to oxidative removal subsequent to an enolic shift to oxygen. Thus the observed activity of (XXVI) may well be the result of a partial dehydrogenation to the vitamin. In sharp contrast to the behavior of the 5,8-dihydride, hexahydrovitamin K_1 (XXVII) gave only a feeble response at the high level of 1000 γ. Fernholz, MacPhillamy, and Ansbacher (47) observed no activity at twice this dosage by the 6 hour method. The substance should be compared with the β,γ-dihydride, for which the effective dose is 8 γ, and it is evident that complete saturation of the non-quinonoid ring produces a profound change. Chemically,

TABLE X

Hydrides of Vitamin K_1 and of Methylnaphthoquinone

Compound	Effective dose
	γ
5,8-Dihydrovitamin K_1 (11, 14) .	4
$\beta,\gamma,5,6,7,8$-Hexahydrovitamin K_1 (11, 15)	1000 (Very slight)
2-Methyl-5,8-dihydro-1,4-naphthohydroquinone (14)..... .	6
2-Methyl-5,8,9,10-tetrahydro-1,4-naphthoquinone (14)..... . .	8
2-Methyl-5,6,7,8-tetrahydro-1,4-naphthoquinone (15)......... .	500

the compound is to be regarded as a benzoquinone with four alkyl substituents, and from this point of view it is most nearly comparable with the inactive 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone ((XVI) above). When the side ring contains no activating double bond, it does not share the susceptibility to dehydrogenation characteristic of compounds of the type (XXVI). The lack of significant activity thus appears to be attributable to the inability of the substance to undergo biological transformation into a naphthoquinone.

Similar relationships were observed among various hydrides of 2-methyl-1,4-naphthoquinone. The condensation of butadiene with toluquinone affords the 5,8,9,10-tetrahydride (XXVIII), which in the presence of a trace of an acid or a base is smoothly

isomerized to 2-methyl-5,8-dihydronaphthohydroquinone (XXIX).
The latter compound was assayed as such rather than in

(XXVIII)   (XXIX)
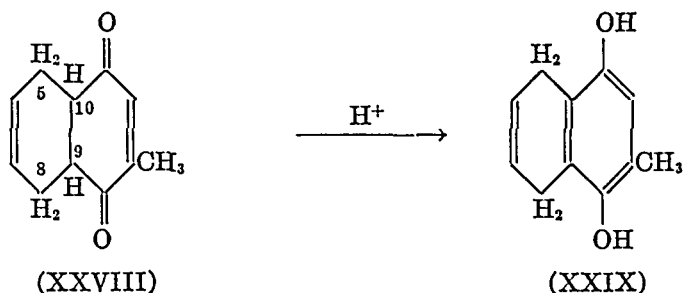
the form of the quinone, of which it is doubtless the biological
equivalent, because the quinone is a highly sensitive compound
subject to rapid deterioration. The substance does not show
as high a ratio of potency to methylnaphthoquinone as does the
5,8-dihydride of vitamin K_1 to the vitamin, possibly because of the
destruction of a part of the sensitive quinone formed as an inter-
mediate in the process of aromatization of the end ring. As in the
vitamin series, the 5,6,7,8-tetrahydride of methylnaphthoqui-
none shows activity of only a low order (compare Fernholz, Mac-
Phillamy, and Ansbacher (47)). The isomeric 5,8,9,10-tetra-
hydride (XXVIII), however, possesses considerable potency,
being about 1/8 as active as vitamin K_1. This diene addition prod-
uct does not possess the structural characteristics and properties
of a quinone and is quite far removed from a naphthoquinone
type. An explanation of the vitamin K activity on the basis of
the structure as such is far to seek, and the biological action of
the compound seems, rather, to be a manifestation of the step-
wise conversion of the substance into methylnaphthoquinone, and
possibly to a true vitamin K type principle.

Naphthalene and Tetralin Derivatives

In a further study of possible biochemical changes 2-methyl-
1-naphthol (XXX) and 3-methyl-1-naphthol (XXXI) were
examined for comparison with 2-methyl-1,4-naphthohydroquinone
(XXXII), which is effective at practically the same level as the
quinone. The two methylnaphthols were found to possess
striking antihemorrhagic potency, giving a full response at 1 γ
and at 0.6 γ, respectively (Table XI). It would seem remarkable

(XXX)   (XXXI)   (XXXII)
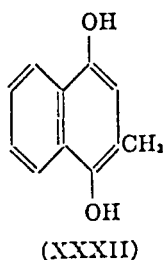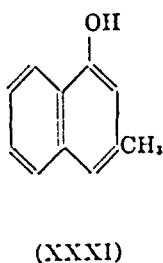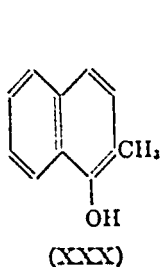
that these simple naphthalene derivatives should be capable of
exerting in their own right an action characteristic of complex

TABLE XI

Methylnaphthols, Methyltetralones, and Related Compounds

Compound	Effective dose
	γ
2-Methyl-1,4-naphthohydroquinone .	0.5
2-Methyl-1-naphthol (12, 15).. . ..	1
3-Methyl-1-naphthol (12, 15)	0.6
4-Methyl-1-naphthol (12, 15).	Inactive at 1000
1-Methyl-2-naphthol (12, 15).. ...	" " 1000
3-Methyl-2-naphthol (12, 15). .	" " 1000
1-Naphthol.... .... , .	1000 (Slight)
2-Methyl-1-tetralone (12, 15) .. .	0.6
3-Methyl-1-tetralone (12, 15)...	1
2-Methyl-1-naphthylamine (12, 15)	5
β-Methylnaphthalene	1000 (Slight)
α-Methyl-γ-phenylbutyric acid (15) . ..	Inactive at 1000

vitamin principles of highly specific structures. 1-Naphthol
evokes no such response. In order to determine whether this is
a general property associated with methylnaphthols we investi-
gated the three other possible isomers having the two groups in
the same ring, (XXXIII) to (XXXV). These are all completely
inactive, and yet they do not depart greatly in structure from the
highly potent isomers, 4-methyl-1-naphthol having the reactive
α-hydroxyl group characteristic of these compounds, and 3-
methyl-2-naphthol the methyl group at the β position. There is,
however, a striking differentiation in this series of five isomers in
that two of them are convertible by direct processes into 2-methyl-
1,4-naphthoquinone and the other three are not. It can hardly
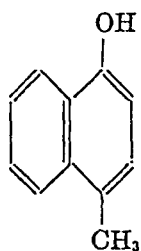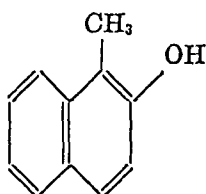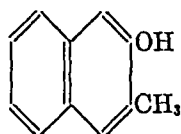
(XXXIII)   (XXXIV)   (XXXV)

be a coincidence that those two which can yield the quinone share its biological action and that the non-convertible isomers are inactive. The results thus provide a strong indication that the active methylnaphthols when administered to animals undergo efficient hydroxylation at the position para to the hydroxyl group.

2-Methyl-1-naphthylamine (XXXVI) shows considerable activity but is only 1/5 as potent as the corresponding methylnaphthol (XXX). The amine may conceivably undergo deamination to (XXX) or para-hydroxylation and oxidation, and in either case
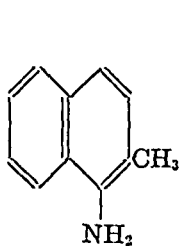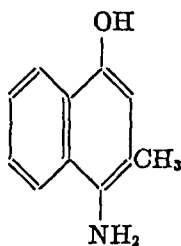
(XXXVI)   (XXXVII)

the over-all process must involve some losses. The *p*-hydroxy compound (XXXVII) has been assayed by Emmett, Kamm, and Sharp (48) and found nearly as potent as methylnaphthoquinone, whereas the methylnaphthylamine is only about 1/17 as effective as this reference substance. A *p*-aminonaphthol probably requires no special process of deamination, since this can occur spontaneously following oxidation of the substance to a quinoneimine.

The results for the tetralones (XXXVIII) and (XXXIX) in Table XI show that these substances give every indication of functioning as precursors of the corresponding methylnaphthols, for they are effective at practically the same level. There is a
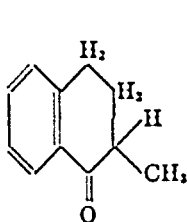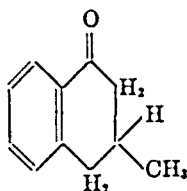
(XXXVIII)   (XXXIX)

slight difference in potency between the two tetralones and the order is the reverse of that of the methylnaphthols. Differences in the relative efficiencies of the various steps in the conversion and in the susceptibility to side reactions would account for such minor variations.

There is no inconsistency in the view that the alicyclic ring of a tetralone undergoes aromatization in the body, whereas that present in 2-methyl-5,6,7,8-tetrahydro-1,4-naphthoquinone does not, for the tetralone can function in the enolic form in which a double bond is supplied to the ring in question and only 2 hydrogen atoms have to be removed in order to produce a phenol. The energy required for aromatization of a tetrahydrobenzenoid ring is far greater than for a dihydride with the bond structure of the enol (XL). The aromatization of a tetralone is thus com-
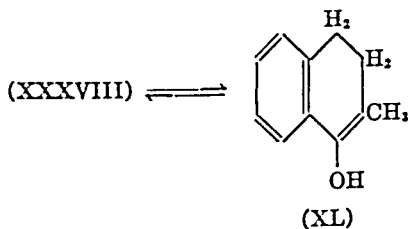
(XL)

parable with that indicated as occurring biologically with the 5,8-dihydronaphthoquinones mentioned.

As far as our observations go, the limit of biological conversion, as indicated by high antihemorrhagic activity, is reached with the tetralones and methylnaphthols. An acid precursor which yields one of the tetralones on cyclodehydration was tested with negative results, and β-methylnaphthalene showed little indication of undergoing biological oxidation.

SUMMARY

Bioassays for antihemorrhagic activity in chicks by the 18 hour method are reported for 79 compounds synthesized or selected with the view of obtaining information on the relationship between vitamin K activity and structure. A number of interesting relationships are suggested by the data, and, although the nature of the phenomenon is such that any interpretation must be tempered with reservation, the following main conclusions seem indicated.

Antihemorrhagic activity of any biologically significant magnitude is found only in the series of the 1,4-naphthoquinones, or among compounds convertible into such quinones. Considerable specificity is evident in the series which embraces vitamins K_1 and K_2 and all other 1,4-naphthoquinones having a methyl group at the 2 position and some more extended group at position 3. A double bond in the β,γ position of the 3-substituted side chain contributes to the potency of the compound, while unsaturation at points more remote from the quinonoid nucleus is without influence. A branched chain isoprenoid structure of the 3-substituent is more favorable for the development of antihemorrhagic activity than a straight chain arrangement, and the carbon content is also a factor of importance. In the series of quinones having a branched side chain the activity tends to increase as the chain is lengthened and reaches a maximum when the chain includes some 20 or 30 carbon atoms.

Although the methyl group at position 2 is important for the development of highest potency, β-alkenylnaphthoquinones lacking this group retain an average of about 1/65 the activity of the methylated quinones and exhibit all the above features of specificity. Any extension of the 2-methyl group, however, or any alkyl substitution in the benzenoid ring, results in complete obliteration of the activity. The high potency of 2-methyl-1,4-naphthoquinone is similarly almost completely wiped out by replacing the methyl group by ethyl or propyl, or by introducing a methyl group at any of the four positions in the benzenoid ring. Hydroxyl substitution in nuclear positions of methylnaphthoquinone or into the side chain of β,γ-dihydrovitamin K_1 results in loss in potency.

The antihemorrhagic activity observed with a considerable number of the compounds tested seems to be attributable not to

the functioning of the actual substance administered but to its conversion in the animal body to a vitamin K type principle in the course of the assay. It appears possible that methylnaphthoquinone exhibits high potency by virtue of its functioning as a component in the biosynthesis of a quinone of the general type exemplified by vitamins K_1 and K_2. Inorganic and organic esters of methylnaphthohydroquinone and vitamin K_1 hydroquinone most likely owe such activity as they exhibit to a more or less efficient hydrolysis in the organism, whereas ether derivatives may be convertible into the corresponding quinones by a process of metabolic oxidation. Still more extensive changes which appear from the assay data to be of likely occurrence are illustrated in the accompanying chart. A figure is included for each compound giving the effective dose in micrograms as found in our assays, and the arrows indicate postulated metabolic changes. Thus the remarkably potent, methyltetralones are considered to suffer dehydrogenation to the methylnaphthols, which in turn

2-Methyltetralone-1
(0.6 γ)

3-Methyltetralone-1
(1 γ)

2-Methyl-1-naphthylamine
(5 γ)

−2H

−2H

2-Methyl-1-naphthol
(1 γ)

3-Methyl-1-naphthol
(0.6 γ)

[O],
H_2O

[O]

[O]

2H

2-Methyl-1,4-naphthoquinone ⇌ 2-Methyl-1,4-naphthohydroquinone
(0.3 γ)   (0.5 γ)

Phytol

Oxide
(1.2 γ)

2H

Vitamin K_1
(1 γ)

−2H

5,8-Dihydride
(4 γ)
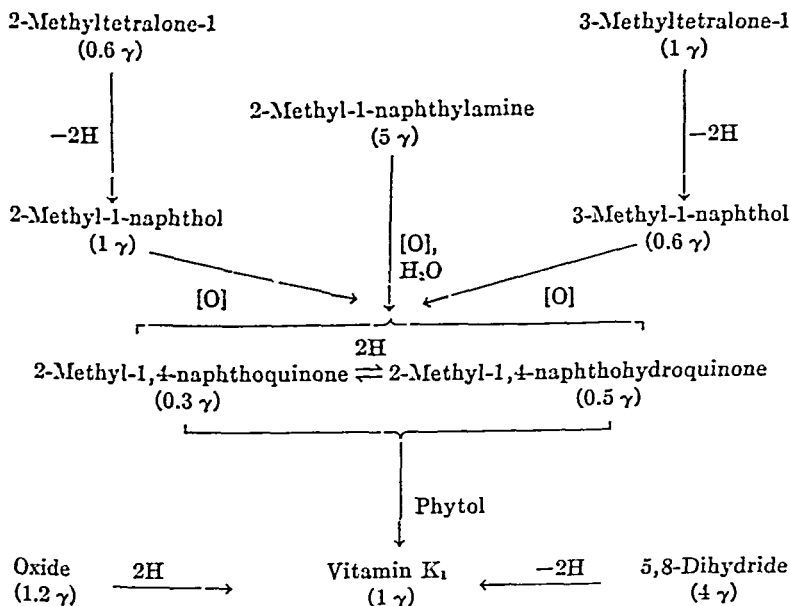
are converted by hydroxylation into methylnaphthohydroquinone; the latter substance, or the biologically equivalent quinone, may then combine with an isoprenoid alcohol to give a vitamin K

factor. The potencies observed for all of the compounds in this complex series do not depart very widely from molecular equivalence. Other biological processes indicated as likely by the assay results apparently proceed with varying efficiency. Thus the oxide of vitamin K_1 is almost equivalent to this substance in activity and therefore may be subject to nearly quantitative bioreduction, whereas that of methylnaphthoquinone probably is in part dissipated by conversion into the less active phthiocol. Compounds having a dihydrobenzenoid ring, as exemplified by 5,8-dihydrovitamin K_1, appear capable of moderately efficient dehydrogenation, but the corresponding tetrahydrides are resistant to such a change.

Addendum (*January 15, 1941*)—Since this paper was submitted for publication Lee, Solmssen, Steyermark, and Foster (49) have published additional assay data for sodium 2-methyl-1,4-naphthohydroquinone diphosphate which are in close agreement with the earlier results from the same laboratory (42) and with the present results (Table VIII). Lee *et al.* are in error in stating that Fieser and Fry (10) reported assays indicating that sodium 2-methyl-1,4-naphthohydroquinone diphosphate is only moderately active. The·statement which they quote refers to the phosphate ester of vitamin K_1 hydroquinone and not to that of 2-methyl-1,4-naphthohydroquinone; the exact dose level of the latter ester had not at the time been determined.

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THE RÔLE OF THE EXTERNAL SECRETION OF THE PANCREAS IN LIPID METABOLISM

THE PREVENTION OF FATTY LIVERS IN DEPANCREATIZED AND DUCT-LIGATED DOGS BY THE DAILY FEEDING OF FRESH PANCREATIC JUICE*

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There can be no doubt that the pancreas contains a factor other than insulin that prevents the deposition of excessive amounts of fat in the liver. Evidence for this view was first obtained in the completely depancreatized dog kept alive with insulin, in which fatty livers were found to develop when the animal was maintained on a diet adequate in proteins, vitamins, and salts but lacking raw pancreas (1-3). Later work showed that ligation of the pancreatic ducts was sufficient to induce fatty livers (4-6). The question now arises as to how the factor in the pancreas that inhibits the accumulation of excessive amounts of fat in the liver is made available to the organism. Although observations on the duct-ligated dog suggest that the factor is released by the external secretion of the gland, the fact must not be overlooked that this procedure is associated with atrophy of a portion of the gland and hence may be responsible for the destruction of an internal secretory mechanism. It would appear that unequivocal evidence for the action of the external secretion could be obtained only by directly examining the effects of the ingestion of pancreatic juice upon lipid metabolism. This has been done in the present investigation.

Sixteen adult dogs were employed in this study; eight of these were depancreatized and eight subjected to duct ligation (4). Before these operations, each dog received twice daily at 8 a.m. and at 4 p.m. a mixture containing 30 gm. of lean meat and 6 gm. of sucrose per kilo. Vitamin and salt supplements were added to the mixture daily in the form of 3 cc. of cod liver oil,[1] 5 cc. of galen B (vitab, Type II, liquid),[2] and 2 gm. of Cowgill's salt mixture (8).

Three of the depancreatized dogs served as controls. They were injected twice daily with insulin and fed a diet of lean meat, sucrose, bone ash, and vitamin supplements. The other five received raw pancreas in addition to the insulin and dietary treatment until they regained a vigorous appetite. The feeding of raw pancreas was then discontinued and pancreatic juice added to the dietary mixture. The control dogs were sacrificed for liver examination 20 weeks after pancreatectomy, the other five at intervals of 23 to 28 weeks after it, during the last 20 weeks of which four of them received an average of 377 cc. and one of them 422 cc. of pancreatic juice daily. The 20 week interval of observation was adopted, since it was previously shown that at least 16 weeks were required to insure the deposition of fatty acids in excess of 14 per cent in the livers of depancreatized dogs receiving a diet containing no raw pancreas (9).

Following duct ligation, the eight dogs received insulin[3] (4) and a diet of lean meat, sucrose, and raw pancreas as well as the vitamin and salt supplements. When the animals had fully recovered from the operation (as shown by the complete healing of the wound and the acquisition of vigorous appetites), the feeding of raw pancreas was discontinued. For 20 weeks thereafter four of the animals received pancreatic juice (422 cc. per dog

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[1] Standardized cod liver oil was kindly furnished by Mead Johnson and Company.

[2] Kindly furnished by Vitab Products, Inc., Emeryville, California. Galen B (vitab, Type II, liquid) is essentially an aqueous extract of rice bran. Its vitamin content is as follows: thiamine 150 γ per cc. (50 u.s.p. units), riboflavin 10 γ per cc., pyridoxine 150 γ per cc., pantothenic acid 400 γ per cc., nicotinic acid 2 mg. per cc. In addition it contains at least two unidentified factors, the eluate factor and the filtrate factor (7).

[3] The insulin was generously donated by Eli Lilly and Company.

per day), whereas the four control dogs were fed only the lean meat-sucrose mixture.

Supply of Pancreatic Juice—Pancreatic juice was obtained from dogs by means of a modified Elman-McCaughan fistula (10) In order to provide adequate amounts of pancreatic juice, it was found necessary sometimes to maintain at one time as many as 9 dogs with fistulae. In all, over 60 juice-producing animals were used in the present study. The longest functional activity of such animals was somewhat over 2 months, the average about 3 weeks. They were maintained on a diet of lean meat and raw pancreas, supplemented with salts and vitamins. Each animal also received intravenously 1 to 2 liters of Ringer's solution daily.

The interval between the collection of pancreatic juice and its administration varied. The juice ingested by Dogs D-228, D-229, D-230, and D-234 was about 16 to 28 hours old, whereas that received by Dogs D-256, E-56, E-63, E-65, and E-66 was about 8 to 10 hours old.

The livers were removed in the morning after the animals had been sacrificed by means of an intravenous injection of 25 mg. of nembutal per kilo of body weight. At this time the dogs were in the postabsorptive state, having received their last meal and injection of insulin at about 4.00 p.m. of the previous day. The whole liver was thoroughly ground and mixed and samples taken for analyses. The oxidative procedures employed for lipid analyses have been described elsewhere (11). Free cholesterol was measured in an acetone solution after the phospholipids had been precipitated.

Results[4]

Depancreatized Dogs—The three control dogs possessed fatty livers when examined 20 weeks following pancreatectomy. The total fatty acid content varied from 15.1 to 20.5 per cent. The data in Table I show that pancreatic juice fed twice daily is a most effective agent in preventing the deposition of excessive amounts of lipid in the liver. In three of the dogs treated with pancreatic juice the total fatty acid content of the liver did not exceed 4 per cent; the fourth had a value of 7 per cent (Dog D-229).

[4] A preliminary report of some of the data has appeared (*Proc. Soc. Exp. Biol. and Med.*, **43**, 349 (1940)).

The ingestion of pancreatic juice resulted in the maintenance of the cholesterol content of the livers within the normal range (3), whereas in the absence of pancreatic juice both the free and the esterified fractions were increased. The phospholipid content of the livers of the dogs receiving pancreatic juice did not differ significantly from that found in the livers of the control dogs.

<div align="center">TABLE I</div>

<div align="center">Influence of Ingestion of Pancreatic Juice upon Liver Lipids of Depancreatized Dogs Maintained with Insulin</div>

The values are expressed as per cent of wet tissue. Each dog after pancreatectomy was fed 500 gm. of meat and 100 gm. of sucrose per day in addition to raw pancreas and pancreatic juice.

Dog No.	Dog weight		Raw pancreas fed		Pancreatic juice fed		Period since pancreatectomy	Liver weight	Cholesterol		Total fatty acids	Phospholipid
	Preoperative*	Final							Total	Free		
	kg.	kg.	wks.	gm. per day	wks.	cc. per day	wks.	gm.	per cent	per cent	per cent	per cent
D-228	10.5	9.5	8	250	20	377	28	530	0.19	0.18	3.70	2.12
D-229	9.9	9.8	8	250	20	377	28	540	0.19	0.14	7.00	2.35
D-230	10.1	9.0	8	250	20	377	28	570	0.21	0.15	5.79	2.25
D-234	16.7	11.1	4	250	20	377	24	535	0.22	0.19	3.11	2.50
D-256	13.6	13.3	3	250	20	420	23	470	0.34	0.30	3.19	1.95
D-208	12.5	12.5					20	1100	0.38	0.30	20.40	2.06
D-209	15.0	7.8					20	500	0.41	0.32	15.10	1.98
D-210	10.2	7.7					20	637	0.36	0.30	20.50	2.55

* The preoperative diets of these dogs consisted of 30 gm. of lean meat and 6 gm. of sucrose per kilo of body weight per day. 2 gm. of Cowgill's salt mixture (8), 3 cc. of cod liver oil, and 5 cc. of vitamin B concentrate were fed daily.

Duct-Ligated Dogs—Three of the control dogs possessed fatty livers, the fatty acid contents of which ranged from 16.6 to 31.4 per cent. The liver of Dog E-59, also a control dog, contained 7.1 per cent fatty acid. It is evident from Table II that the daily ingestion of pancreatic juice is just as effective a control in the duct-ligated dog as in the depancreatized dog. The highest value for total fatty acid observed in the four dogs that received pancreatic juice daily did not exceed 5 per cent.

An interesting difference between the depancreatized and the

duct-ligated dogs was found in the response of their body weights. Despite the feeding of pancreatic juice, the former failed in most cases to maintain their preoperative weights, but all the duct-ligated animals that received pancreatic juice showed a gain in weight.

TABLE II

Influence of Ingestion of Pancreatic Juice upon Liver Lipids of Duct-Ligated Dogs Maintained with Insulin

The values are expressed as per cent of wet tissue.   Each dog after duct ligation was fed 500 gm. of meat and 50 gm. of sucrose per day in addition to the raw pancreas and pancreatic juice; during a period of 3 weeks each received 250 gm. of raw pancreas per day.   The control dogs were fed at 8.00 a.m. and at 4.00 p.m.   The juice-fed dogs were fed at 8.00 a.m. and at 9.00 p.m.

Dog No.	Dog weight		Pancreatic juice fed		Period since duct-ligation	Liver weight	Cholesterol		Total fatty acids	Phospholipids
	Pre-operative*	Final					Total	Free		
	kg.	kg.	wks.	cc. per day	wks.	gm.	per cent	per cent	per cent	per cent
E-56	12.1	16.0	20	420	23	350	0.24	0.21	2.24	2.37
E-63	15.0	19.7	20	420	23	290	0.25	0.23	4.90	2.92
E-65	14.3	17.0	20	420	23	317	0.26	0.18	4.98	4.17
E-66	13.0	18.0	21	420	24	372	0.21	0.21	2.62	2.66
E-57	10.8	10.7			23	255	0.22	0.21	31.40	3.46
E-59	9.2	7.3			23	268	0.26	0.24	7.13	2.36
E-64	15.3	9.2			23	550	0.41	0.24	16.60	1.27
E-70	14.8	10.5			23	381	0.30	0.20	22.70	1.56

* The preoperative diets consisted of 30 gm. of lean meat and 6 gm. of sucrose per kilo of body weight per day.   2 gm. of Cowgill's salt mixture (8), 3 cc. of cod liver oil, and 5 cc. of vitamin B concentrate were fed daily. 5 gm. of bone ash were given with each meal.

DISCUSSION

The results presented here provide conclusive evidence that fatty livers produced either by excision of the pancreas or by duct ligation can be completely prevented by the daily ingestion of fresh pancreatic juice.   This observation establishes an essential function for the *external secretion of the pancreas*; namely, the maintenance of a normal lipid level in the liver.   It would appear that the daily release of pancreatic juice into the gastrointestinal

tract is necessary for inhibiting excessive amounts of fat from being deposited in the liver.

The mechanism whereby pancreatic juice brings about this effect is at present obscure. Absorption of the liver factor probably occurs after the pancreatic juice is poured into the small intestine, but an answer to the question whether it is absorbed unaltered in the form present in the pancreatic juice or whether before absorption it is changed by an interaction with the intestinal mucosa or some dietary constituent must await further investigation. Moreover, it is still not impossible that pancreatic juice may be only indirectly involved, and that some substance in either the lumen or the mucosa is acted upon by the pancreatic juice to produce the active factor. Irrespective of these speculations, it is now apparent that the evidence provided by Van Prohaska, Dragstedt, and Harms (12) for the claim that the liver factor is released by the pancreas by way of the blood stream only and not through the external secretion of the gland is no longer tenable.

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THE RÔLE OF THE EXTERNAL SECRETION OF THE PANCREAS IN LIPID METABOLISM

THE INFLUENCE OF DAILY INGESTION OF PANCREATIC JUICE UPON THE LEVEL OF BLOOD LIPIDS IN COMPLETELY DEPANCREATIZED AND DUCT-LIGATED DOGS MAINTAINED WITH INSULIN*

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The observation that the maintenance of a normal lipid content in the liver is dependent upon the daily supply of an adequate amount of the external secretion of the pancreas (1) suggested a similar investigation with regard to the control of lipids in the blood. Previous studies have shown that a fall in all blood lipid constituents occurs in completely depancreatized dogs kept alive with insulin and in dogs in which the pancreatic ducts have been ligated when pancreas replacement therapy is withheld (2, 3). The daily ingestion of raw pancreas was found to have a unique effect in the diabetic dog in that it not only prevented the fall in lipids but led to an increase in the lipid concentration of the blood markedly in excess of the normal (4). The blood lipids of the duct-ligated dog were also maintained at high levels by the feeding of raw pancreas (3). This property of *raising the blood lipids above normal* was lost when the raw glandular tissue was autoclaved, although in large amounts it still possessed the curative action on the liver and the ability to raise the blood lipid to or near the normal or preoperative level (5). This limited action

on the blood lipids is similar to that produced by the ingestion of large amounts of choline (6).

<center>EXPERIMENTAL</center>

The dietary treatments employed for the maintenance of the completely depancreatized and duct-ligated dogs and the procedures by which large volumes of pancreatic juice were supplied over a period of several months have been recorded in the preceding paper (1).

Whole blood was removed for analyses while the animals were in the postabsorptive state. The oxidative procedures employed for the determination of total cholesterol, phospholipid, and total fatty acids have been described elsewhere (2). Free cholesterol was measured in the acetone solution after the phospholipid had been precipitated.

<center>*Results*[1]</center>

Depancreatized Dogs—The blood lipids of each of five dogs (Table I) were determined during three experimental states: before pancreatectomy; during a period of 3 to 8 weeks after pancreatectomy, when the dogs were fed raw pancreas (in addition to the lean meat-sucrose diet); and during a subsequent 20 weeks, when the daily feeding of fresh pancreatic juice was substituted for the raw glandular tissue.

A comparison of the values obtained during these three states indicates that pancreatic juice is as effective as raw pancreas in raising the blood lipids of the depancreatized dog above preoperative levels. This is particularly well shown in Dog D-230. Before pancreatectomy the values for total cholesterol were 150 to 155 mg. per 100 cc. of whole blood. At the end of the 8 week period after pancreatectomy, during which raw pancreas was fed daily, total cholesterol had risen to 240 mg., the increase being reflected in both the free and the esterified fractions. During the 20 weeks in which pancreatic juice was administered, the blood lipids of this animal were examined seven times. Six of the values for total cholesterol were well over 200 mg., one being as high as 277 mg. In two of the dogs (Nos. D-228 and D-229) some fluctuations were

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[1] A preliminary report of some of the data has appeared (*Proc. Soc. Exp. Biol. and Med.*, **40**, 6 (1939)).

TABLE I

Effect of Ingestion of Pancreatic Juice for Long Intervals upon Whole Blood Lipids of Completely Depancreatized Dogs Maintained with Insulin

Dog No.	Weight	Period after pancreatectomy	Period fed pancreas	Period receiving pancreatic juice	Cholesterol			Total fatty acids	Phospholipid	Total lipid
					Total	Free	Ester			
	kg.	wks.	wks.	wks.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
D-228	10.5	(1)*			142	128	14	368	315	510
	10.5	(0)			153	138	15	363	313	516
	9.9	5.8	5.8†		176	138	37	356	442	531
	9.6	8.0	8.0		198	148	50	452	402	650
	9.2	10.4		1.9‡	166	94	72	412	336	578
	9.2	12.7		4.2	182	121	61	382	382	564
	9.1	15.4		6.9	151	85	66	308	361	459
	9.5	18.4		9.9	142	85	57	410	327	552
	9.7	19.4		10.9	165	106	59	376	365	541
	9.5	23.7		14.2	157	103	54	422	399	579
	9.5	28.0		20.0	175	110	65	453	400	628
D-229	9.9	(1)			170	128	42	380	313	550
	9.9	(0)			161	132	29	393	320	554
	9.7	5.8	5.8		214	154	60	471	416	685
	9.7	8.0	8.0		232	172	60	463	363	695
	9.5	10.4		1.9	232	166	66	448	445	680
	9.4	12.7		4.2	235	163	72	463		698
	9.4	15.4		6.9	175	141	34	475	376	650
	9.5	18.4		9.9	175	153	22	322	346	497
	9.4	19.4		10.9	177	116	61	321	361	498
	9.6	23.7		14.2	170	96	74	338	338	508
	9.8	28.0		20.0	151	92	59	403	388	554

* Before pancreatectomy each dog received daily 30 gm. of meat and 6 gm. of sucrose per kilo of body weight supplemented with 2 gm. of Cowgill's salt mixture (7), 3 cc. of cod liver oil, and 5 cc. of vitamin B concentrate. The numbers in parentheses refer to the number of weeks *before* pancreatectomy.

† During the interval when raw pancreas was fed each animal received twice daily 125 gm. of raw pancreas and 250 gm. of lean meat.

‡ When the feeding of pancreatic juice was introduced, each animal received twice daily 250 gm. of lean meat and 50 gm. of sucrose in addition to the pancreatic juice. Dogs D-228, D-229, D-230, and D-234 were fed at 8.00 a.m. and at 4.00 p.m. Dog D-256 was fed at 8.00 a.m. and at 9.00 p.m. The first four dogs received an average of 377 cc. of pancreatic juice daily, whereas the last dog received an average of 420 cc. daily.

TABLE I—*Concluded*

Dog No.	Weight	Period after pancreatectomy	Period fed pancreas	Period receiving pancreatic juice	Cholesterol			Total fatty acids	Phospholipid	Total lipid
					Total	Free	Ester			
	kg.	*wks.*	*wks.*	*wks.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
D-230	9.7	(1.7)			150	127	23	404	372	554
	10.1	(0)			155	141	14	389	348	544
	9.2	8.0	8.0		240	172	68	496	460	738
	8.9	10.4		1.9	234	147	87	526	435	760
	8.8	12.7		4.2	277	151	126	576	508	853
	9.0	15.4		6.9	238	123	115	545	356	783
	9.1	18.4		9.9	184	102	82	518	395	702
	9.0	19.4		10.9	239	116	123	452	324	691
	9.1	23.7		14.2	215	132	83	438	477	653
	9.0	28.0		20.0	224	107	117	508	492	732
D-234	16.3	(1)			178	132	46	491	450	669
	16.7	(0)			184	132	52	527	422	711
	13.9	4	4.0		193	133	60	564	420	757
	12.0	6		2.0	222	124	98	550	480	772
	11.4	8		4.0	192	122	70	545	442	737
	12.2	14		10.0	194	112	82	558	408	752
	12.1	15		11.0	200	110	90	593	317	793
	12.3	19		15.0	202	113	89	562	342	764
	11.1	24		20.0	191	126	65	446	446	637
D-256	14.2	(2.5)			143	112	31	338	372	481
	14.7	(1)			146	106	40	311	358	457
	13.6	3	3		226	128	98	530	413	756
	13.5	7		4	210	117	93	524	487	734
	14.5	11		8	226	108	118	480	473	706
	14.6	14		11	205	109	96	482	371	687
	14.3	16		13	235	125	110	534	492	769
	13.3	23		20	159	105	54	419	383	578

noted, and although certain of the cholesterol values found during the feeding of pancreatic juice were in close agreement with those observed during the preceding interval, in which raw pancreas was fed, this was not always the case. Despite similar irregularities in phospholipid and total fatty acids, there can be no doubt, from a comparison of the values obtained in the three intervals studied, that pancreatic juice is capable of maintaining elevated levels of these lipid constituents.

Duct-Ligated Dogs—The blood lipids of four dogs (Table II)

TABLE II
Effect of Ingestion of Pancreatic Juice on Blood Lipids of Duct-Ligated Dog

Dog No.	Weight	Period after duct ligation	Period fed pancreas	Period fed pancreatic juice	Cholesterol			Total fatty acids	Phospholipid	Total lipid
					Total	Free	Ester			
	kg.	*wks.*	*wks.*	*wks.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
E-56	12.1	(1)*			201	125	76	425	402	626
	12.1	(0)			176	108	68	470	392	646
	12.5	3	3†		172	107	65	421	332	593
	13.5	6		3‡	185	129	56	423	432	608
	14.4	11		8	233	140	93	393	489	626
	14.7	15		12	195	109	86	470	398	665
	15.8	22		19	205	130	75	505	486	710
	16.0	23		20	220	124	96	549	460	769
E-63	15.0	(1)			153	116	37	388	460	541
	15.0	(0)			160	114	46	409	417	569
	15.4	3	3		189	122	67	498	384	687
	15.0	7		4	164	127	37	466	441	630
	18.1	11		8	196	141	55	448	382	644
	19.6	22		19	207	124	83	432	474	639
	19.7	23		20	199			473	425	672
E-65	14.3	(1)			186	96	90	456	382	642
	14.3	(0)			186	95	91	474	343	660
	14.0	3	3		161	94	67	362	344	523
	15.0	6		3	171	106	65	392	352	563
	15.9	10		7	200	115	85	423	451	623
	16.5	15		12	170	99	71	491	492	661
	17.0	23		20	189	115	74	400	427	589
E-66	13.0	(1)			159	101	58	445	355	604
	13.3	3	3		178	107	71	451	405	629
	15.0	7		4	180	107	73	422	406	602
	16.5	11		8	182			351	430	533
	17.3	15		12	169	118	51	444	382	613
	18.0	21		18	174	114	60	482	421	656
	18.0	23		20	201	126	75	515	481	716

* The numbers in parentheses refer to the number of weeks *before* duct ligation. Before duct ligation each animal received 30 gm. of lean meat and 50 gm. of sucrose per kilo per day plus vitamin supplements with each evening meal. After duct ligation all dogs received twice daily a diet consisting of 250 gm. of lean meat and 25 gm. of sucrose plus vitamin supplements twice weekly. Each dog received 8 units of insulin after each meal.

† During the interval when raw pancreas was fed each dog received 125 gm. of the raw gland with every meal.

‡ When pancreatic juice was fed, each dog received an average of 420 cc. per day in addition to the lean meat-sucrose diet. They were fed at 8.00 a.m. and at 9.00 p.m.

were examined during three periods of observation: before duct ligation; during the first 3 weeks after duct ligation, in which raw pancreas was fed; during a subsequent period of 20 weeks, in

TABLE III

Influence of Ingestion of Various Amounts of Pancreatic Juice upon Whole Blood Lipids of Completely Depancreatized Dogs Maintained with Insulin

All dogs received 250 gm. of meat, 50 gm. of sucrose, and 5 gm. of bone ash per meal plus vitamins twice weekly. 8 units of insulin were given after each meal.

Dog No.	Weight	Period of observation	Pancreatic juice		Cholesterol			Total fatty acids	Phospholipid	Total lipid
			Interval fed juice	Average amount per day[*]	Total	Free	Ester			
	kg.	*days*	*days*	*cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
D-196	7.6	0	0		127	97	30	308	337	435
	8.2	25	25	60	166	89	77	410	363	576
	7.7	40	.0		115	105	10	294	321	409
D-155	7.4	0	0		94	86	8	246	183	340
	8.5	25	25	100	137	86	51	382	349	519
	7.9	40	0		97	82	15	236	279	333
D-198	7.6	0	0		153	135	18	382	328	535
	8.1	22	22	250	235	171	64	563	433	798
	7.5	38	0		132	123	9	336	280	468
D-101	8.5	0	0		109	97	12	247	142	356
	8.9	27	27	250	185	119	66	427	356	612
	8.6	42	0		108	108		233	228	341
D-104	6.5	0	0		121	116	5	280	183	401
	7.0	19	19	300	205	120	85	425	403	630
	6.5	47	0		124	117	7	360	234	484
D-196	7.7	0	0		172	110	62	296	262	468
	8.4	27	27	315	211	145	66	501	441	712
	7.7	42	0		120	99	21	325	285	445

[*] Amounts recorded fed in two equal parts at 8.00 a.m. and at 4.00 p.m.

which the feeding of pancreatic juice was substituted for the raw glandular tissue. A comparison of the levels of cholesterol, phospholipid, and total fatty acid found during these three intervals shows that a factor that inhibits the fall of all these lipid constituents is contained in the external secretion of the pancreas.

The values for cholesterol, phospholipid, and total fatty acid found at various intervals after the feeding of pancreatic juice had been instituted were as high as, or higher than, the preoperative values. The elevation above normal observed in the depancreatized dog is not so evident in the duct-ligated dog.

Effects of Various Amounts of Pancreatic Juice upon Blood Lipids —In Table III are shown the results of an attempt to determine the smallest amount of pancreatic juice that will raise the blood lipids. Before the administration of the pancreatic juice, the six depancreatized dogs were maintained upon a diet of lean meat and sucrose, to which vitamin and salt supplements had been added. As pointed out elsewhere, this diet results in a fall in the level of all lipid constituents of the blood. To test the effects of small amounts of pancreatic juice, it seemed desirable to use dogs in which the blood lipids were low, for it is to be expected that such dogs would more readily respond to the factor raising the lipids than dogs in which the lipids had been maintained at a high level for some time. For this reason depancreatized dogs that had been kept on a diet deprived of pancreas until the blood lipids had fallen to low levels were used. The blood lipids of each dog were determined three times, (1) before the administration of pancreatic juice was started, (2) at the end of a period of 19 to 27 days in which pancreatic juice was fed daily, (3) at the end of a subsequent 15 to 28 day period during which no pancreatic juice was fed.

Amounts of pancreatic juice as small as 60 cc. daily (30 cc. with each meal) were found effective in raising the lipid level of the blood. With this dose of pancreatic juice, significant changes in esterified cholesterol and in total fatty acids of the blood were noted (Dog D-196). Higher doses (50 cc. per meal to Dog D-155) produced decided responses in esterified cholesterol, phospholipid, and total fatty acids. Increases in free cholesterol were observed when daily feedings of 250 cc. or more were employed. In all cases the cessation of pancreatic juice feedings resulted in a drop in the level of cholesterol, phospholipids, and total fatty acids.

SUMMARY

The ability to raise the cholesterol, phospholipid, and total fatty acid content of the blood previously shown to be contained in raw pancreas is demonstrated in the present study to be associ-

ated with the external secretion of the gland. The daily ingestion of pancreatic juice was found to be effective in raising the low levels created by lack of replacement therapy and in maintaining the normal levels of cholesterol, phospholipid, and total fatty acid in the blood of completely depancreatized and duct-ligated dogs maintained with insulin.

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COMBINATION OF PROTEINS AND METAPHOSPHORIC ACID

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The addition of an excess of metaphosphoric acid to egg albumin has been shown to lead to the formation of a precipitate containing a constant proportion of bound metaphosphate (16). Protein and precipitant apparently combine in stoichiometric proportions. Furthermore, the value for bound metaphosphate appears to agree with the acid-combining capacity of the protein as determined by electromotive force measurements (3, 4).

The present study indicates that a similar relation holds for other proteins. The uniformity of the relationship suggests moreover that precipitation by metaphosphoric acid may be used as an analytical procedure to determine the total acid-combining capacity of proteins.

EXPERIMENTAL

The following six proteins, chosen since they represent proteins with a wide range of chemical and physicochemical properties, were selected for study: (1) crystalline zinc insulin, kindly supplied to us by Eli Lilly and Company; (2) crystalline edestin, kindly supplied by Dr. H. B. Vickery; (3) crystalline horse hemoglobin, prepared according to the method of Ferry and Green (5); (4) crystalline horse serum albumin, three times recrystallized, carbohydrate-free, prepared according to the method of McMeekin (13); (5) pseudoglobulin γ_1, prepared by Dr. Arda A. Green; (6) myosin (rabbit), prepared by a slight modification of the method of Greenstein and Edsall (7). All the proteins, except the crystalline zinc insulin and edestin, were prepared in this laboratory. The metaphosphoric acid was the commercial c. p. Baker preparation.

The experimental technique was the same as that previously described. Protein solutions containing usually 10.0 to 15.0 mg. of protein nitrogen in 5.0 cc. were mixed with a sufficient amount of a freshly prepared solution of 0.1 N metaphosphoric acid to adjust the reaction to an acidity greater than pH 2.0. This high acidity was necessary in order to repress completely the

TABLE I

Precipitation of Proteins with Metaphosphoric Acid

Protein	Protein solution		Metaphosphate ppt.		pH of filtrate
	Total N per cc. protein solution	Total P per cc. protein solution	Total N	Total P	
	mg.	*mg.*	*mg.*	*mg.*	
Insulin T1161	2.64	None	2.52	0.54	1.32
			2.53	0.54	
" 972519	2.24	"	2.22	0.53	1.60
			2.16	0.52	
Edestin	3.22	"	3.18	0.73	1.54
			3.17	0.72	
Hemoglobin (horse)	2.10	"	2.09	0.58	1.72
			2.095	0.57	
	2.48	"	2.35	0.63	1.94
			2.45	0.65	
Serum albumin (horse)	2.79	"	2.74	0.77	1.81
			2.72	0.75	
Pseudoglobulin (horse)	2.35	"	2.03	0.35	1.60
			2.08	0.37	
Myosin (rabbit)	0.58	0.0199	0.52	0.162	1.69
			0.51	0.162	
	0.46	0.0079	0.44	0.133	
			0.45	0.132	

ionization of the positively charged groups of the protein. The precipitate formed was separated by centrifugation and washed carefully until the washings contained neither protein nor phosphorus. Each precipitate was then dissolved in concentrated sulfuric acid, brought to a volume of 10.0 cc., and analyzed for nitrogen and phosphorus. The nitrogen was estimated by the Pregl micro-Kjeldahl method, phosphorus by the method of Lohmann and Jendrassik (12).

Results

The analytical data are summarized in Table I. The second and third columns give the nitrogen and phosphorus in the protein solutions analyzed; the fourth and fifth in the separated metaphosphoric precipitates. Two different preparations of

TABLE II

Comparison of Acid-Combining Capacity

	N	Moles acid per gm. protein $\times 10^5$, estimated from			
		E.M.F. measurements	Content of basic amino acids (arginine, lysine, histidine)	Other measurements	Bound metaphosphoric acid
	per cent				
Egg albumin	15.6	80 (3) 87 (4)	76.3 (18)	61 (14) 75 (15)	78 (16)
Insulin	14.28	101 (8)	85.6 (20)	112 (15) 93 (11)	101 110
Edestin	18.64	127 (2) 134 (10)	124.7 (18)	151 (14)	138
Hemoglobin (horse)		148 (6)	125.3 (19)		148 150*
Serum albumin (horse)	16.0	140†	140.3 (9)		140
Pseudoglobulin γ_1 (horse)	15.57	92‡			89
Myosin (rabbit)	16.7	155§	119.6 (17)		149 150
Clupein	22.6		390 (21)	410 (15)	384 (16)

The figures in parentheses refer to bibliographic references.

* Herrmann, H., unpublished.
† Cohn, E. J., McMeekin, T. L., and Blanchard, M. H., unpublished.
‡ Cohn, E. J., Green, A. A., and Blanchard, M. H., unpublished.
§ Edsall, J. T., and Gale, A., unpublished.

insulin, hemoglobin, and myosin were studied and each analysis was carried out in duplicate. The results thus reveal the reproducibility of the method both with different samples of the same preparation and with different preparations of the same protein.

In Table II the averages of these results expressed as the ratio of metaphosphate to protein are compared with data upon (1) the acid-combining capacity of proteins, (2) the basic amino

acid content of proteins, and (3) the complex formation of proteins with chondroitinsulfuric acid and with inorganic complex salts. In each case, the values for acid-combining capacity from metaphosphate are in excellent agreement with values derived from electromotive force measurements and in general slightly higher than the results thus far available for the sum of the basic amino acids (18). Since these results obtained by isolation or analysis after hydrolysis of the proteins are quite possibly a little low, this discrepancy may be regarded as a reasonable one. The agreement among the figures obtained by these different procedures, especially for the electromotive force measurements and metaphosphate-combining capacity, can be regarded as additional evidence that the latter method may be generally applicable to proteins.[1]

SUMMARY

A variety of proteins combine with metaphosphoric acid to form an insoluble precipitate in which the phosphorus content is equivalent to the number of positively charged groups of the protein.

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[1] In apparent contradiction of our findings Briggs (1) claims that no maximum value of bound metaphosphate is reached when the ratio $PO_3:NH_2 = 1$. To explain this discrepancy we should like to point out that Briggs' method of determining "bound metaphosphate" is entirely different from that here described. In it the value is calculated from titration data in a system containing besides the protein a variety of inorganic forms. It is very likely that under these conditions the reaction has a more complex character. We therefore attempted to isolate one part of this system and by direct analysis determined the metaphosphate content of the precipitate obtained when an excess of metaphosphoric acid is added to a protein solution. Under these conditions, in the cases mentioned above, the stoichiometric relation holds.

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METABOLISM OF KIDNEY TISSUE IN THE ADRENALECTOMIZED RAT*

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An important problem in the study of the rôle of the adrenal cortex in metabolism is the determination of the relationship between the aberrations in electrolyte and water distribution and the defects in protein and carbohydrate metabolism which have been shown to occur in adrenalectomized animals. A point of attack is presented by the observation (1) that the kidney of the adrenalectomized dog maintained in good condition on extra sodium chloride and a low potassium intake fails to concentrate potassium in the urine to a normal extent. Since the kidney fails to do work efficiently, one may expect to find underlying defects in the energy-yielding processes of the tissue. This expectation is realized in the experiments described below. Kidney tissue slices from adrenalectomized rats have a lower rate of oxygen uptake than normal rat kidney slices, both in the absence and in the presence of substrates. The rate of ammonia production from amino acids is also less than normal. These defects can be repaired by the administration of adrenal cortical extract or of desoxycorticosterone to the adrenalectomized animals.

Methods

Oxygen consumption was measured by the Warburg manometric technique. The main room of each vessel contained 2.5

ml. of the physiological saline solution containing phosphate buffer (pH 7.4) described by Krebs (2). The concentration of substrate was 0.01 M, the temperature of the bath was 37.5°, and the vessels were filled with oxygen. The experimental animals were male albino rats of Wistar strain, weighing 120 to 200 gm. Adrenalectomized rats were never used sooner than a week after operation, and they were given a 1 per cent sodium chloride solution to drink. They were inspected after death to check the completeness of the adrenalectomy. All animals were fasted overnight (14 to 17 hours) before being used in the experiments.

The tissues were prepared by the following standardized procedure. The animal was anesthetized with nembutal injected intraperitoneally, the abdomen was opened, and one kidney was excised after ligation of its vessels and washed in physiological saline. The organ was bisected and each half was quickly sliced by hand (3) into uniform thin slices which were collected in a Petri dish containing physiological saline. The remaining kidney was then removed and dealt with in the same way. Three slices from the pooled lot were placed in each of the Warburg vessels which had been prepared for the experiment. 0.2 ml. of 30 per cent potassium hydroxide was placed in the central well of the vessels, and they were attached to their manometers, filled with oxygen, and placed in the bath. 10 minutes shaking were allowed for temperature equilibration, after which measurements of oxygen uptake were made at 15 minute intervals for $1\frac{1}{2}$ hours. At the end of this time 0.5 ml. of N hydrochloric acid was tipped in from the side bulbs and a further 10 minute shaking period was allowed. The vessels were then dismounted and 0.3 ml. of 25 per cent trichloroacetic acid was added to each vessel. The slices were removed, washed, and put into tared vials for drying to constant weight at 110°. When ammonia was to be determined, 0.5 ml. of 40 per cent sodium hydroxide was put in the side bulb of each vessel, and the vessels were mounted in a multiple aeration apparatus. The receivers, 15 ml. test-tubes containing 3 ml. of 0.05 N sulfuric acid, were connected up, the alkali was tipped into the main room of the vessels, and brisk aeration was maintained for 45 to 50 minutes. The fluid in the receivers was analyzed for ammonia by Van Slyke's manometric hypobromite method (4). It was found that a hypobromite solution made up

with 5 per cent sodium hydroxide produced fewer gas bubbles and was less tenacious of them than the solution recommended by Van Slyke. Recoveries of known small amounts of ammonia by this method were perfectly satisfactory.

There are three important points in this procedure. (1) The use of an anesthetic and the maintenance of the circulation in the tissue until the last possible moment make it easier to wash the tissue free of blood. (2) The time elapsed between the removal of the tissue and the first measurement of oxygen uptake varied within narrow limits, so that from one experiment to another the same period in the history of the tissue after its removal from the animal was studied, and differences due to a decline in the activity of the tissues with time were largely avoided. (3) The aeration of the residual fluid in the vessels reduces the amount of manipulation and the attendant possibility of loss of the small amounts of ammonia present in the reaction mixture.

Kidney tissue was taken from three groups of animals, normal rats, adrenalectomized rats maintained on sodium chloride, and adrenalectomized rats in a state of acute insufficiency, and the rates of oxygen uptake were determined in the absence of substrate and in the presence of the substrates dl-alanine, pyruvic acid, $l(+)$-glutamic acid, α-ketoglutaric acid, succinic acid, and citric acid. The rates of ammonia production were determined in the absence of substrate and in the presence of the two amino acids. The effects of the injection of adrenal cortical extract and of desoxycorticosterone were studied in two groups of normal animals and in three groups of adrenalectomized rats maintained on sodium chloride.[1] The routine of the injection of cortical extract was as follows: On the evening of the 2nd day before the experiment 1 ml. of cortical extract was injected intraperitoneally into the rat, and this was repeated in the morning, at noon, and in the evening of the following day. During this period the animal had access to food and drink, but it was fasted as usual on the night before the experiment. On the next morning the rat was

given one more injection, about an hour before the experiment. This routine is called the "short treatment." The "long treatment" differed from it only in that beginning at 8 o'clock in the evening of the day before the experiment the rat received 1 ml. of cortical extract every hour for the next 12 hours. In every instance, the treated adrenalectomized rats gained at least 10 gm. in weight during the first 24 hours of treatment, and at the time of the experiment the blood sugar level was at or above the normal level. The routine of the injection of desoxycorticosterone followed that of the "short treatment." The compound was injected subcutaneously, in a fine suspension in 25 per cent glucose solution, in amounts such that the animals received 2.5 to 5.0 mg. of desoxycorticosterone over the whole period.

Since the data were derived from a large number of animals, they have been evaluated with the primary consideration in mind that any differences observed should be due to real differences between the tissues, and not merely to chance differences between individual animals. It was found that the variations between individual tissue samples from the same animal were larger than the variations in the mean response of the tissue samples from different animals. The uniformity of behavior of tissue derived from different animals makes it possible to treat each tissue sample as an individual observation and to pool all the observations in an experimental series in order to obtain the best estimate of the response of the tissue to the experimental conditions. The necessary statistics for obtaining these estimates have been calculated from the data by the methods outlined by Fisher (5).

In tissue slice experiments the activity of tissues is customarily expressed as c.mm. of substance metabolized per mg. of dry weight of tissue per hour, the Q value, but before such a term may be used it should be shown that there is a satisfactory linear relationship between the rate of the metabolic process measured and the dry weight of the tissue (6) and that this relationship is not qualitatively different in the kidney tissue from normal and adrenalectomized rats. Assurance was gained on these points by calculating the regression of the rate of oxygen uptake upon the dry weight of tissue, in the absence of substrate, for the groups of observations from the normal rats, adrenalectomized rats that

were salt-maintained, and adrenalectomized animals in a state of acute insufficiency. The regression formulae found were

Normal................................ $Y = 19.33x + 7.94$
Adrenalectomized, salt-maintained....... " $= 16.45x + 25.16$
           "   insufficient .... " $= 16.00x + 3.82$

where Y is the rate of oxygen uptake in c.mm. per hour and x is the dry weight of the tissue in mg. The plotted points are distributed uniformly about straight lines passing through, or nearly through, the origin (0 c.mm. of oxygen; 0 mg. of dry weight), and the term Q_{O_2} may therefore be used to compare the residual rates of oxygen uptake of kidney tissue from normal and adrenalectomized rats. Since the regressions were derived from samples taken from many animals, they offer additional evidence of the uniformity of behavior of the tissue.

When the same relationship was plotted for the observations made in the presence of the substrates, it was found that the straight lines of best fit calculated from the data were a group of nearly parallel lines which intercepted the Y axis at a considerable distance from the origin. These regression lines were apparently rather poor approximations to fairly uniform portions of a family of curved lines. In the presence of the substrates used, therefore, the relationship between rate of oxygen uptake and dry weight of the tissue, for kidney slices of both normal and adrenalectomized rats, is best expressed by a curved line beginning at the origin and decreasing in slope with increase in dry weight. The term Q_{O_2} must in these circumstances be limited, in describing the response of tissue to a substrate. In order that the mean rates of oxygen uptake of several groups of tissue samples may be compared, the mean dry weights of the several groups must be the same, or differ only within narrow limits, since the Q_{O_2} of any group is the slope of the tangent to the curved regression line at the mean dry weight of the series of samples, and wide differences in the mean dry weight will tend either to minimize or to exaggerate any differences that may exist between groups of observations. Care has been taken in these experiments to insure a reasonable uniformity in the mean dry weight in the groups of tissue samples that are to be compared with one another. In the few instances

in which this was not possible, the differences in the mean dry weight are such that differences in the rate of oxygen uptake tend to be minimized. As an additional precaution the rate of oxygen uptake in the absence of substrate was determined with at least two samples of tissue from every animal, so that a good measure of the condition of the tissues in each experiment was obtained. In Tables I to III, the rates of oxygen uptake and of ammonia production are the mean rates for the 1½ hour period of the experiment, expressed as c.mm. per mg. of dry weight per hour.

Results

Table I summarizes the data on oxygen uptake from the several groups of experiments. Acute adrenal insufficiency is accompanied by a marked decrease in the residual oxygen uptake of the kidney slices. Although maintenance of the adrenalectomized animals with sodium chloride raises the residual respiration nearly to the normal level, there is no corresponding improvement in the response of the tissue to *dl*-alanine and pyruvic acid. Sodium chloride does not repair the fundamental defect in the oxidative metabolism of kidney tissue from adrenalectomized rats. The rate of oxidation of every substrate, except citric acid, by kidney slices from rats in chronic adrenal insufficiency is significantly less than normal. The probability that these differences could have occurred by chance is 1 in 100 or less, and, since the mean dry weights of the several groups correspond closely, the differences may be considered real. The possibility that these differences may be due to diminished viability of tissue as a consequence of adrenalectomy is examined in Fig. 1, in which the oxygen uptake, in c.mm. per hour, is plotted for each successive ½ hour period of the experiments with each substrate and for both classes of tissue. There are no outstanding differences in the course of the respiration in the two classes of tissue. Diminished viability of tissue after adrenalectomy does not contribute to the differences observed, although some such contribution might have been expected if there is any basis for the idea, often expressed, that in addition to its specific metabolic effects, the hormone of the adrenal cortex exercises a general beneficent influence upon tissue cells.

Treatment of adrenalectomized animals with cortical extract

TABLE I

Effects of Adrenalectomy, Adrenal Cortical Extract, and Desoxycorticosterone upon Rates of Oxygen Uptake of Tissue Slices of Rat Kidney

The values represent $-Q_{O_2}$ measured in c.mm. of O_2 per mg. of dry weight per hour. The figures in parentheses indicate the number of observations in each group.

	No substrate	dl-Alanine	Pyruvic acid	l(+)-Glutamic acid	α-Ketoglutaric acid	Succinic acid	Citric acid
Normal	20.24 ± 0.21 (85)	27.55 ± 0.39 (58)	30.18 ± 0.43 (48)	32.92 ± 0.53 (65)	29.21 ± 0.45 (26)	38.01 ± 0.78 (29)	27.62 ± 0.35 (29)
Adrenalectomized, on salt	19.36 ± 0.24 (90)	25.39 ± 0.32 (58)	26.23 ± 0.28 (60)	29.45 ± 0.38 (55)	26.55 ± 0.38 (26)	34.01 ± 0.62 (30)	27.08 ± 0.28 (60)
Adrenalectomized, acutely insufficient	16.51 ± 0.36 (18)	24.23 ± 0.78 (6)	26.22 ± 0.89 (11)				
Adrenalectomized, cortical extract, short treatment	20.68 ± 0.47 (49)	32.53 ± 0.45 (46)	29.50 ± 0.43 (29)	33.53 ± 0.71 (18)	30.03 ± 0.45 (29)		
Adrenalectomized, cortical extract, long treatment	20.51 ± 0.28 (14)	32.96 ± 0.84 (8)	31.64 ± 0.36 (18)	33.27 ± 0.51 (20)		38.33 ± 0.53 (20)	
Normal, cortical extract, short treatment	21.46 ± 0.36 (18)	33.97 ± 0.45 (9)	30.87 ± 0.58 (10)	36.31 ± 0.81 (28)	29.21 ± 0.63 (10)		
Adrenalectomized, desoxycorticosterone	20.35 ± 0.28 (24)		29.06 ± 0.53 (16)	31.97 ± 0.39 (31)			
Normal, desoxycorticosterone	22.38 ± 0.38 (24)		31.23 ± 0.00 (10)	33.05 ± 0.46 (31)			

or with desoxycorticosterone restores the rates of oxidation by the tissue slices to or above the normal levels, and the similar treatment of normal rats tends to increase the rates above normal levels.   It may be significant, in view of the effects of the cortical hormone upon protein metabolism (7), that the increases are most marked in the presence of the two amino acids.   This point may also be related to the improvement in the residual rate of respiration of the slices, since it has been suggested that the marked ability of the kidney to deaminate amino acids and the fact that
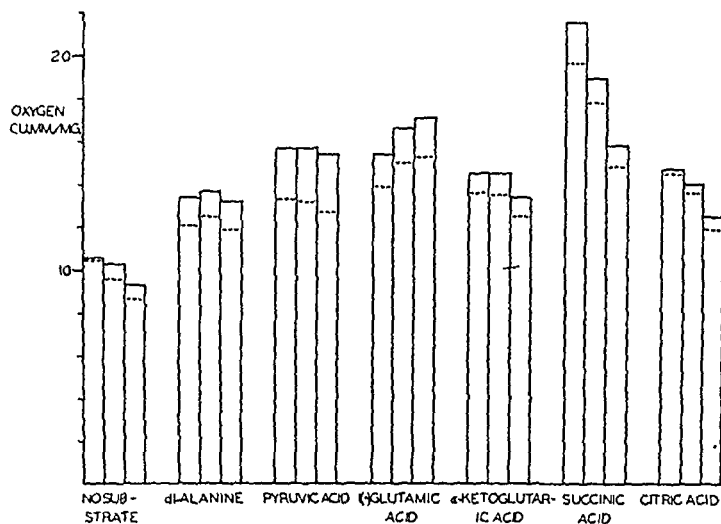
FIG. 1. The course of the respiration of kidney slices of normal and adrenalectomized rats.   Each column represents a ½ hour period of the experiments.   The solid horizontal lines indicate the levels of oxygen uptake for the normal tissue, and the broken lines are the levels for the kidney tissue of adrenalectomized rats.

the respiratory quotient of kidney tissue lies between 0.8 and 0.9 indicate that the main substrate being oxidized in kidney may be protein.   The long treatment with excessive amounts of cortical extract does not produce exaggerated increases in rates of oxidation, which suggests that the amount of cortical hormone in this system is not a limiting factor in the rate at which these processes may take place.   Desoxycorticosterone appears to be as effective as the cortical extract in maintaining normal or supernormal rates of oxidation in the kidney tissue.   Attempts to demonstrate

a similar action of cortical extract *in vitro* have so far been uniformly unsuccessful, but so long as the site and mode of action of the adrenal cortical hormone are unknown, such negative results are not critical, except to indicate that the proper conditions of experiment have not been established.

Table II summarizes the data on ammonia production from the several groups of experiments. There is a small production

TABLE II

Effects of Adrenalectomy, Adrenal Cortical Extract, and Desoxycorticosterone upon Rates of Ammonia Production in Tissue Slices of Rat Kidney

The values represent $+Q_{NH_3}$ measured in c.mm. of ammonia per mg. of dry weight per hour. The figures in parentheses indicate the number of observations.

	No substrate	dl-Alanine	l(+)-Glutamic acid
Normal	1.93 ± 0 12 (55)	6 97 ± 0 29 (55)	5 77 ± 0 17 (63)
Adrenalectomized, on salt	1 54 = 0 12 (52)	5 80 ± 0 19 (41)	4 02 ± 0 14 (55)
" acute insufficiency		6 02 ± 0 76 (6)	
Adrenalectomized, cortical extract, short treatment	2 00 ± 0 13 (20)	7 56 ± 0 20 (42)	4 80 = 0 18 (17)
Adrenalectomized, cortical extract, long treatment	1 30 ± 0 24 (5)	7 75 ± 0 44 (9)	5 68 ± 0 27 (18)
Normal, cortical extract, short treatment	2 49 ± 0 21 (7)	8 00 ± 0 23 (9)	7 38 = 0 31 (28)
Adrenalectomized, desoxy-corticosterone	1 81 ± 0 14 (15)		4 43 ± 0 15 (28)
Normal, desoxycorticosterone	1 55 ± 0 11 (16)		5 45 ± 0.18 (29)

of ammonia by kidney slices in the absence of substrate, probably derived in part from the breakdown of protein and the deamination of the amino acid residues. A portion of it is preformed ammonia, in the sense that it is produced by the tissue during its preparation for experiment. A number of observations were made upon kidney slices prepared for experiment in the usual manner, but treated with strong acid at the end of the 10 minute equilibration period. The Q_{NH_3} of twelve such control samples

averaged 1.30 ± 0.17, which indicates that a large proportion of the ammonia found in the absence of substrate was present before the 1½ hour experimental period had begun. It is therefore difficult to attribute any particular significance to the residual ammonia production, or to lay emphasis upon differences between groups of observations. Thus, although the individual values for the residual ammonia production have been given in Table II, the mean value of the 170 observations, 1.79 ± 0.06, probably offers a better estimate of a base-line from which to measure the effects of the addition of the amino acids.

The rate of ammonia production in the presence of the two amino acids is significantly less in kidney tissue from rats in adrenal insufficiency than it is in normal kidney slices. In each instance, the probability that the differences might have occurred by chance is less than 1 in 100. The magnitude of the differences is such that they account for only a part of the differences in the rate of oxygen uptake in the presence of the amino acids reported in Table I, and this is to be expected, since there are also differences in the rate of oxidation of the corresponding keto acids. Treatment of the adrenalectomized rats with cortical extract or with desoxycorticosterone restores the rates of ammonia production to, or nearly to, the normal level, and the treatment of normal animals has produced a slight increase in rates of ammonia production in two out of three instances. Rat kidney tissue is capable of synthesizing glutamine (8), a fact which may explain the small residual ammonia production in this tissue, and which also indicates that the estimates of rate of deamination in Table II, as well as the differences in rates, are likely to be minimal estimates and differences. The apparent low rate of deamination of glutamic acid may also be due to the synthesis of glutamine.

Since the rate of removal of the products of deamination might be expected to influence the rate of deamination of an amino acid, it is possible that the observed differences in rate of ammonia production are due to differences in the rate of oxidation of the keto acid formed. In the presence of sodium arsenite the further oxidation of keto acids is inhibited, so that this poison may be used to determine whether there is in fact a real difference in rates of deamination of amino acids in the two classes of tissue. Accordingly, two series of observations were made upon kidney slices from normal and adrenalectomized rats, treated with 0.001

м sodium arsenite. The rates of oxygen uptake and ammonia production, in the absence of substrate and with *dl*-alanine, are presented in Table III. In the absence of substrate the rates of oxygen uptake and ammonia production in the two series are not significantly different. These observations have been pooled to provide a better estimate of the base-line from which the effects of the substrate may be measured. With *dl*-alanine the rates of ammonia production are of the same order of magnitude as those presented in Table II, and there is a significant difference between

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TABLE III

Rates of Oxygen Uptake and Ammonia Production from Kidney Slices, Treated with 0.001 м Sodium Arsenite, of Normal and Adrenalectomized Rats

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The figures in parentheses in Column 2 indicate the number of observations in each group. N. = normal; A. = adrenalectomized.

Substrate (1)	Tissue (2)	$-Q_{O_2}$ (3)	Increment (4)	Normal minus adrenalectomized (5)	Q_{NH_3} (6)	Increment (7)	Normal minus adrenalectomized (8)	Ratio (7)/(4) (9)	Ratio (8)/(5) (10)
None	N. (12)	4.79 ±0.22			1.23 ±0.16				
	A. (12)	4.73 ±0.21		0.06 ±0.30	1.15 ±0.11		0.08 ±0.19		
	N. + A. (24)	4.76 ±0.15			1.19 ±0.09				
dl-Alanine	N. (12)	7.35 ±0.52	2.59 ±0.54		7.02 ±0.63	5.83 ±0.64		2.25	
	A. (10)	6.25 ±0.52	1.49 ±0.54	1.10 ±0.73	4.41 ±0.41	3.22 ±0.42	2.61 ±0.76	2.16	2.37

the rates in the two series. The ratios of increment in ammonia production to increment in oxygen uptake for the two series, and the ratio of the differences in these increments between the two series, have been calculated. Since 2 moles of ammonia correspond to 1 mole of oxygen in the deamination reaction, the ideal ratio is 2.0, and in view of the difficulty of measuring low rates of oxygen uptake with accuracy, the correspondence of the calculated ratios to the ideal is satisfactory. There is, then, a real decrease in the rate of deamination of the amino acids by the kidney tissue of adrenalectomized rats.

The evidence presented in this paper confirms earlier work in two respects: first, that the rate of deamination of amino acids by kidney tissue from adrenalectomized animals is less than normal (9), and second, the recent observation (10) that kidney slices from rats in acute adrenal insufficiency exhibit a residual oxygen uptake about 25 per cent below that of normal kidney slices. These observations are extended by the demonstration that the rates of oxidation of certain keto acids, and of succinic acid, are also less than normal after adrenalectomy. The action of adrenal cortical extract and of desoxycorticosterone in restoring these rates to normal levels or better is strong evidence of the relation of the defects in oxidation to the absence of the cortical hormone. The evidence supports the proposition that the failure of the kidney to deal adequately with electrolytes after adrenalectomy may be related to an underlying failure in the energy-yielding processes of the kidney. Against the possible objection that the magnitude of the differences observed is out of all proportion to the drastic final consequences of adrenalectomy three considerations are offered. First, the rat, a small animal with a high metabolic rate, requires 5 to 10 days to die of adrenal insufficiency, even after a long period of maintenance on sodium chloride. Second, the kidney is not the only organ affected; there is a failure in the work performance of voluntary muscle (11) and of glyconeogenesis in the liver (7). Third, it is possible that in a coordinated series of metabolic processes the failure of one or more in moderate degree may be critical in determining the success of the over-all process. The bulk of the available evidence indicates that the failures of metabolic processes in adrenalectomized animals are not absolute, but are failures in degree; it is when such animals are required to supply or to expend energy at accelerated rates that they collapse.

There is no indication at present of the nature of the changes which take place in kidney tissue after adrenalectomy. It has been reported that the concentrations of flavin-adenine dinucleotide (12) and of cozymase (13) in rat kidney tissue are not significantly altered after adrenalectomy. It appears that these two enzyme factors are not affected in adrenal insufficiency, but there are many more which may be investigated. If the results of such an investigation are uniformly negative, the problem may

nevertheless be attacked from the point of view that the hormone of the adrenal cortex is responsible for the coordination of enzyme systems for the performance of special tasks in the kidney. Further work has been planned and is in progress on both these lines, and in addition certain functions of the kidney in the intact adrenalectomized rat are being studied in the light of the results presented in this paper.

SUMMARY

The rates of oxygen uptake, in the absence of substrate, and in the presence of the substrates *dl*-alanine, pyruvic acid, *l*(+)-glutamic acid, α-ketoglutaric acid, and succinic acid are significantly less than normal in kidney slices from adrenalectomized rats. The rates of deamination of the two amino acids, as judged by the ammonia production, are also less than normal. These activities of kidney tissue from adrenalectomized rats may be restored to normal levels or better by the administration to the adrenalectomized animals of adrenal cortical extract or of desoxycorticosterone.

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THE EFFECT OF DEFICIENCIES IN COPPER AND IRON ON THE CATALASE ACTIVITY OF RAT TISSUES*

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The necessity of copper for the synthesis of hemoglobin has stimulated studies on a possible effect of copper on the formation of other heme compounds by living organisms. Most prominent among the catalytically active heme compounds described thus far are the cytochromes, cytochrome oxidase, catalase, and peroxidase. It is also of interest to determine how a deficiency of iron affects the relative concentration of these compounds in various tissues.

Elvehjem (1) found that bakers' yeast grown on a purified medium low in iron was deficient in the cytochromes, whereas inclusion of iron in the medium yielded a yeast with a more intense cytochrome spectrum. Addition of copper enhanced the spectral bands of cytochrome a. Yoshikawa (2) obtained similar results and found in addition that the catalase activity of yeast grown on an iron-deficient medium was below normal. From their studies of tissues from anemic rats Cohen and Elvehjem (3) concluded that copper deficiency leads to a decrease of the cytochrome a content of the tissues and to reduced indophenol oxidase activity. Cytochromes b and c were apparently not affected by deficiencies in iron and copper. The recent work of Keilin and Hartree (4) with heart muscle preparations indicates that the spectral α band previously ascribed to cytochrome a is a composite of cytochrome a and another heme compound (perhaps cytochrome

oxidase). In view of these observations the effect of copper on cytochrome *a* in living organisms may require reinvestigation.

Quantitative determinations have established that copper deficiency causes a great reduction of cytochrome oxidase activity in liver and heart tissue (5) as well as in the bone marrow[1] of rats and that feeding of copper to deficient rats restores the normal cytochrome oxidase activity of the tissues very quickly.

Catalase from liver has been shown (6) to contain protoheme IX, identical with that of hemoglobin. This made it desirable to investigate the effect of dietary deficiencies in iron and copper on the catalase activity of rat tissues.

EXPERIMENTAL

Young rats were made deficient in copper or iron as described previously (5). After H_2S treatment and concentration the milk was fortified with enough thiamine chloride to insure a daily intake of at least 20 γ of this vitamin per rat. It should also be noted that the iron-deficient rats received at all times 0.1 mg. of Cu plus 0.05 mg. of Mn per day and that the copper-deficient rats received 0.5 mg. of Fe plus 0.05 mg. of Mn per day. The severity of the deficiency was judged by hemoglobin determinations and by the general condition of the animals.

Catalase activity was determined by permanganate titration of the hydrogen peroxide remaining after the enzyme had been allowed to react on an excess of dilute hydrogen peroxide. The conditions of the catalase determination were those outlined by Hennichs (7). All solutions and diluents were cooled in an ice bath and the enzyme activity was determined at this temperature (4°).

Tissues—The tissues from decapitated rats were immediately removed and rinsed. A weighed portion was homogenized (8) with 10 parts of water and the suspension filtered through a cloth. A 2 ml. aliquot was dried at 105° to constant weight. Another aliquot, 2 or 4 ml. depending upon the anticipated enzyme activity, was diluted to 10 ml. in the case of the ventricles and to 50 ml. in the case of liver and kidney. 1 ml. of the dilute enzyme solution was added to an ice-cold mixture of 35 ml. of 0.02 N H_2O_2, 10 ml. of M/15 phosphate buffer of pH 6.8, and 4 ml. of

[1] Schultze, M. O., unpublished results.

water. After exactly 2 minutes (electric timer) 5 ml. of the reaction mixture were discharged into 10 ml. of 1 N H_2SO_4 and titrated with 0.002 N $KMnO_4$. A blank without H_2O_2 was always determined to correct for reducing substances in the tissues. The corrected titration was subtracted from the titration of the H_2O_2 initially present. All determinations were carried out in duplicate. The enzyme activity was calculated for the time t from the rate of reaction according to the equation $(1/t) \ln (C_0/C_t) = k$ and expressed as the conventional *Kat. f.* which represents k per gm. of dry tissue used. (C_0 and C_t represent the concentration of H_2O_2 before and 2 minutes after addition of the enzyme solution respectively.)

Blood—0.04 ml. of blood from the tail was measured with a hemoglobin pipette and discharged into 3.96 ml. of redistilled water. 1 ml. of this solution served as the source of the enzyme and was added to the reaction mixture described above. The rest of the determination was made as outlined. Moisture determinations showed that the per cent water is much higher and more variable in the blood of anemic rats than in the blood of normal rats. Hence the catalase activity of the blood cannot be rationally expressed in the same terms as that of the tissues. For convenience we have calculated catalase activity in terms of mM of H_2O_2 decomposed in 2 minutes by 1 ml. of blood.

The catalase determinations of the blood were made with three litters of rats at weekly intervals. A litter of six rats was made copper-deficient and then treated with 0.1 mg. of copper per rat per day. A litter of seven rats was made iron-deficient and then fed 0.5 mg. of iron per rat per day. A control litter of eight rats received 0.5 mg. of iron plus 0.1 mg. of copper per rat per day at all times.

DISCUSSION

Tissues—Table I summarizes the results of the catalase determinations made with liver, kidney, and ventricles. Quite striking is the fact that both the liver and kidney of male rats possess greater catalase activity than the same tissues of females. Tadokoro and Kyozo (9) have reported similar observations. In tissues of older normal animals of both sexes the catalase activity is somewhat less. No data on catalase activity of rat tissues

TABLE I

Catalase Activity of Rat Tissues

	No. of rats and sex	Time after weaning	Weight	Hb per 100 ml. blood	Kat. f.		
					Liver	Kidney	Ventricles
		wks.	gm.	gm.			
Normal controls	5 ♂	18–19	288	12–13	260 ± 29.4	167 ± 15	9.8 ± 0.8
Milk + 0.5 mg. Fe + 0.1 mg. Cu + 0.05 mg. Mn per day	4 ♀	18–19	202	12–13	143 ± 12.2	115 ± 7.7	10.7 ± 0.7
Normal controls	7 ♂	3–5	117	10–11	286 ± 53	195 ± 14	12.4 ± 1.9
Milk + 0.5 mg. Fe + 0.1 mg. Cu + 0.05 mg. Mn per day	3 ♀	3–5	105	11–12	191 ± 7	142 ± 1	14.8 ± 1.2
Iron-deficient	7 ♂	7–10	124	3–4	209 ± 32.9	94 ± 25.9	9.0 ± 2.4
Milk + 0.1 mg. Cu + 0.05 mg. Mn per day	5 ♀	7–10	114	3–4	148 ± 14.8	97 ± 22.5	10.2 ± 0.6
Copper-deficient	12 ♂	6–7	61	3–4	74 ± 29.6	78 ± 9.8*	21.4 ± 6.4
Milk + 0.5 mg. Fe + 0.05 mg. Mn per day	6 ♀	6–7	60	3–4	102 ± 33.8	87 ± 15.4	24.8 ± 8.8
Same, followed by 0.1 mg. Cu per day for 2 days	3 ♂	6–7	65	3–4	123 ± 32.2	102 ± 6.2	13.3 ± 3.8
	3 ♀	6–7	65	3–4	93 ± 11.4	90 ± 11.4	11.0 ± 1.6
Same, followed by 0.1 mg. Cu per day for 5 days	3 ♂	6–7	74	4–6	215 ± 45.4	138 ± 15.0	16.9 ± 3.3
	7 ♀	6–7	65	4–6	203 ± 23.9	109 ± 7	15.6 ± 4.9

* Average of eleven animals.

have come to our attention which would permit a quantitative comparison with our results.

As a result of iron deficiency the catalase activity of kidney and particularly of liver is reduced. This reduction, however, is less extensive than that of hemoglobin in the blood of the same animals suffering from iron deficiency. The rat evidently has the ability to conserve the concentration of some physiologically active iron compounds in preference to others even when the bodily stores of iron have been exhausted owing to a severe iron deficiency. Whipple and his associates have repeatedly pointed out similar relations in hemorrhagic dogs maintained on diets low in iron.

Much more pronounced than in iron deficiency is the decreased catalase activity of liver and kidney of copper-deficient rats. Although the variations between individual animals are considerable, the reduction of catalase activity of liver and kidney as a result of copper deficiency is clear cut. This is particularly emphasized by the return toward normal catalase values following copper therapy.

Determinations of catalase activity of the ventricles were included in this study because it has previously been shown (5) that the cytochrome oxidase activity of this tissue is markedly decreased in copper deficiency. The catalase activity of ventricular tissue is quite low and it is not decreased in copper deficiency. On the contrary our results indicate an increased catalase activity of the ventricles of copper-deficient rats. We can offer no explanation for this anomaly between the heart and the other tissues studied and the physiological significance of this phenomenon is obscure. It would seem to emphasize, however, the ability of the animal organism to adjust, under stress, the local concentration of catalytically active compounds, provided that means for their synthesis and maintenance are not completely lacking.

Blood—It is well established that blood has a high catalase activity which is confined to the corpuscles. Hemoglobin, however, has only a negligible action on H_2O_2 (10, 11) and cannot account for the activity of the blood. The effect of deficiencies of iron and of copper in rats on the concentrations of hemoglobin and catalase of the blood is similar. In each case the concentration of both heme compounds is decreased during the deficiency and it is rapidly restored upon feeding of the lacking metal (Fig. 1).

The deficiency and recovery curves for catalase and hemoglobin
are not strictly parallel.   This is also true for young growing
rats which, under our experimental conditions, are anemic at the
time of weaning.   During recovery from this anemia, on a diet
of milk supplemented with iron, copper, and manganese, the
catalase activity of the blood reaches a normal level before the
hemoglobin concentration.

The literature contains many observations on the relation
between catalase activity of the blood of various species and its
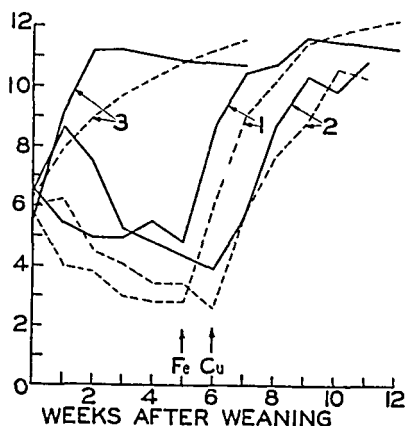
FIG. 1. The effect of iron and copper on the catalase activity of the blood.
The solid lines represent catalase activity expressed as mM of H_2O_2 decom-
posed per ml. of blood in 2 minutes; the broken lines, gm. of hemoglobin
per 100 ml. of blood.   Curves 1, iron-deficient litter fed 0.5 mg. of Fe per
rat per day at the arrow marked Fe, 5 weeks after weaning; Curves 2,
copper-deficient litter fed 0.1 mg. of Cu per rat per day at the arrow marked
Cu, 6 weeks after weaning; Curves 3, normal control litter fed 0.5 mg. of
Fe and 0.1 mg. of Cu per rat per day from the time of weaning.

content of hemoglobin and of erythrocytes.   In general anemia
is associated with reduced catalase activity of the blood and an
increase of erythrocytes and hemoglobin is coincident with a
rising catalase activity of the blood.   No strict parallelism has
been reported between catalase activity of the blood and erythro-
cyte count or hemoglobin content.   The catalase content of the
individual red cells, expressed by the ratio between catalase ac-
tivity and erythrocyte count (catalase index), is not always
constant but usually has been found to increase during anemia

and recovery from it. Such observations were made clinically in various forms of anemia (12–16) and experimentally following hemorrhage (17–21), treatment with phenylhydrazine (17, 20, 21), or exposure to reduced oxygen tension (22, 23).

Sumner and Dounce (24) found that crystalline catalase is essentially free from copper. Agner (25) has withdrawn his earlier intimation (26) that a copper compound may be associated with active preparations of catalase. It appears therefore that the effect of copper deficiency on catalase activity of the rat tissues studied is similar to its effect on the formation of hemoglobin and on cytochrome oxidase activity. Our observations thus add another to the list of heme compounds the synthesis of which is suppressed or impossible in the absence of adequate amounts of copper in the animal organism. If the necessity of copper for the synthesis of heme compounds is a general biological phenomenon, the essential nature of copper for animal life could be explained more rationally.

SUMMARY

1. In experimental copper deficiency the catalase activity of liver, kidney, and blood of rats is markedly decreased. Feeding of copper to the deficient rats induces a rapid return toward normal catalase activity.

2. Iron deficiency also leads to a decreased catalase activity of liver, kidney, and blood.

3. The catalase activity of the heart is not decreased under the same conditions. In copper deficiency it is even increased.

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A SIMPLE APPARATUS AND PROCEDURE FOR DETERMINATION OF AMINO ACIDS BY THE NINHYDRIN REACTION*

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Considerable work has recently been published on the ninhydrin reaction with amino acids (2, 4, 5). This reaction appears to be specific for carboxyl groups of α-amino acids, while other organic acids such as lactic, acetic, and citric are unaffected (4). Because ninhydrin does not react with urea, peptides, other primary amines, and ammonia, Van Slyke and coworkers report this reagent to be more specific for amino acids in biological material than the classical nitrous acid reaction (4).

The utilization of ninhydrin for the quantitative determination of amino acids has been attempted in several ways. Earlier work was confined to colorimetric or photometric procedures which were subject to rather serious limitations (2).

Later Ruhemann (3) and Grassmann and von Arnim (1) have shown that the color-forming reaction of ninhydrin with amino acids is accompanied with the evolution of CO_2.

Both Van Slyke (4) and Dillon and Mason (2) have devised methods for the determination of amino acids based on the measurements of the CO_2 evolved. These investigators have adapted the manometric procedures as developed by Van Slyke (6, 7) and others for the determination of liberated carbon dioxide. This necessitates the use of equipment which may not be readily available in many laboratories.

Recently Christensen has developed a very simple apparatus for the measurement of evolved carbon dioxide which has been successfully applied in several ways (8). Since the apparatus appears to lend itself perfectly to the determination of amino acids by the ninhydrin reaction, this investigation was undertaken.

Method

Apparatus—The apparatus used in this investigation is described in a previous article (8).

Solutions—(1) Barium hydroxide; (2) HCl; (3) thymol blue; (4) glycerol; (5) ninhydrin solution containing 30 mg. of ninhydrin per cc.; (6) saturated KH_2PO_4.

Procedure

The absorption vessel B (*cf.* (8) Fig. 1) is evacuated to approximately 30 mm. and filled with air drawn through a soda lime tower. It is then charged with approximately 2.5 ml. of 0.05 N Ba(OH)$_2$ accurately measured with a protected automatic pipette (8) and again evacuated. This operation should be done as fast as possible. In order to prevent sticking, the ground glass joints are lubricated with glycerol.

1 ml. of water is placed in the U-tube connecting the reaction vessel with the absorption flask. 1 to 4 mg. of amino acid in 2 ml. of water are introduced into the reaction vessel which is then connected to the absorption flask through the U-tube. 1 ml. of ninhydrin solution and 1 ml. of saturated phosphate solution are introduced into the cup of the reaction vessel which is then connected to a large soda lime tower.

The reaction vessel is then brought to a temperature of 110–115° and maintained there for 15 minutes by means of a phosphoric acid bath. During the heating sufficient (CO$_2$-free) air is slowly drawn through the system (by opening slightly the stop-cock of the absorption flask) to maintain the reaction vessel slightly below atmospheric pressure. After heating, the system is permitted to come to atmospheric pressure by opening the stop-cock of the absorption flask so that the flow of air is even and well controlled.

The absorption flask is then disconnected and set aside for 15 minutes. 3 ml. of acetone are now added to the flask and the excess Ba(OH)$_2$ titrated with approximately 0.03 N HCl from a

5 ml. microburette. During the titration the contents of the flask are protected from atmospheric CO_2 by a rubber dam held over the mouth of the flask with a rubber band. The tip of the burette is admitted through a pinhole cut in the center of the cover.

Results

Blank Determinations—Owing to small amounts of contaminating CO_2 (from reagents and residual air) blank runs are necessary

TABLE I

Comparison of Results by Ninhydrin and Micro-Kjeldahl Methods

Amino acid	Weight of sample	Per cent recovery	
		Ninhydrin	Kjeldahl
	mg.		
Glycine..........................	1.848	95.3	101.5
Aspartic acid.....................	1.930	100.2	99.9
Cysteine..........................	2.615	99.5	99.8
Leucine..........................	2.783	100.5	99.8
Norleucine.......................	2.555	.100.7	99.5
α-Aminobutyric acid..............	2.365	100.9	100.3
Phenylalanine....................	2.620	100.8	100.3
Glutamic acid*...................	3.600	100.7	99.9
Valine...........................	2.470	101.0	99.9
Serine...........................	1.763	100.4	100.1
Alanine..........................	1.965	99.4	99.9
Isovaline........................	3.450	1.0	
Tyrosine.........................	4.470	100.3	100.0
Lysine dihydrochloride†..........	3.940	112.5	116.5
Cystine..........................	3.630	96.2	96.7
Tryptophane.....................	1.115	100.1	
α-Aminocaprylic acid.............	3.100	100.1	

* Heated for 5 minutes.
† Calculated on the basis of pure lysine dihydrochloride.

to standardize the procedure. 4.60 ml. of HCl were required to neutralize 2.5 ml. of $Ba(OH)_2$. In blank runs 4.54, 4.54, and 4.52 ml. of acid were required.

Analysis of Amino Acids—In order to check the accuracy of the method, samples of pure amino acids were analyzed by this method and by the micro-Kjeldahl procedure.

As indicated in Table I, alanine, serine, and tryptophane gave

theoretical results. Mason reports low results (2) with these three amino acids when run under slightly different conditions

In two respects the observations of Van Slyke were confirmed. Lysine gave variable results (depending on the acidity). Glutamic acid gave high results if the reaction was allowed to run the usual time.

SUMMARY

1. A simple procedure is described for the determination of amino acids by reaction with ninhydrin.

2. The time required for a complete determination is 20 minutes.

3. Agreement with Kjeldahl determinations of amino acids was within 1 per cent.

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THE CONVERSION OF β-PHENYLLACTIC ACID TO TYROSINE IN NORMAL RATS*

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In a recent publication (1) experiments were described demonstrating the conversion of phenylalanine to tyrosine by normal rats on a stock diet. The phenylalanine added to the diet contained stably bound deuterium, and the rate and extent of the conversion reaction were indicated by the deuterium content of tyrosine isolated from the body proteins. It was shown that the reaction proceeded even when relatively large amounts of tyrosine in addition to the deutero phenylalanine were added to the diet, and it was concluded that the conversion was one of the automatic processes which take place spontaneously and independently of the animal's nutritional requirements for the reaction product.

A similar type of experiment has now been carried out, in which the deutero phenylalanine was replaced by its α-hydroxy derivative, deutero *dl*-phenyllactic acid. Although it is questionable whether the compound is a normal intermediate in amino acid metabolism, it has been shown by Rose (2) to support the growth of rats on a phenylalanine-free diet; while Jervis *et al.* have observed an increase in the output of urinary phenylpyruvic acid (3) and in the phenylalanine content of the blood (4) after feeding phenyllactic acid to humans afflicted with phenylpyruvic oligophrenia. These findings suggested strongly that the hydroxy acid could be converted to phenylalanine, probably via phenylpyruvic acid. It was therefore to be expected that phenyllactic acid might also be converted to tyrosine via phenylalanine, at least in cases of nutritional deficiency of the latter amino acid.

The deutero *dl*-phenyllactic acid was prepared from deutero *dl*-phenylalanine described in the previous studies (1), by treatment with silver nitrite and hydrochloric acid. The compound contained 18.5 ± 0.18 atom per cent deuterium;[1] and as this value coincided with that calculated from the deuterium content of the phenylalanine employed no deuterium had been lost from the α position during removal of the amino group. The distribution of isotope in the molecule must thus have been the same as in the phenylalanine; *i.e.*, it also contained 96 per cent of its isotope

TABLE I

Biological Conversion of Phenyllactic Acid and of Phenylalanine to Tyrosine by Rats

The *dl*-phenyllactic acid added to the diet contained 18.5 atom per cent deuterium.

Deutero substance added to stock diet per rat per day	No. of animals	Average weight of animals	Deuterium content of tyrosine isolated from		Fraction of tissue tyrosine derived from added phenylalanine	
			Internal organs	Rest of body tissues	Internal organs	Rest of body tissues
		gm.	*atom per cent*	*atom per cent*	*per cent*	*per cent*
100 mg. phenyl-lactic acid.....	3	330	0.50 ± 0.01	0.15 ± 0.01	7.0	2.1
150 mg. phenyl-alanine........	2	300	1.48 ± 0 02	0.47 ± 0.02	20.8	6.6

in the phenyl ring. The experiment was performed with a group of three adult rats having an average weight of 330 gm., which remained constant over the experimental period. The animals were maintained for 10 days on the stock diet (5), with an addition of 100 mg. of deutero *dl*-phenyllactic acid per rat per day. At the end of the feeding period the rats were killed, and two samples of tyrosine were isolated, one from the protein of the combined internal organs, and another from the protein of the remaining tissues.

The isolated tyrosine contained considerable quantities of deuterium (Table I) and thus had originated from the hydroxy

[1] The isotope content of the phenyllactic acid is higher than that of the parent phenylalanine (16.8 atom per cent), owing to the loss of 1 non-isotopic hydrogen atom during the conversion.

acid.[2] Deuterium analyses of tyrosine from the corresponding group of animals which received deutero *dl*-phenylalanine (1) are included for comparison. As the isotope distribution in the phenylalanine and phenyllactic acid was the same, the tyrosine derived from the two sources must have had an identical isotope content and distribution. The values in the last two columns of Table I were therefore calculated, as in the previous paper, on the assumption that the tyrosine derived from the experimental compounds contained 7.1 atom per cent deuterium. The amounts of phenylalanine and of phenyllactic acid fed were different but if the figures are reduced to a common basis they seem to indicate that as a tyrosine precursor the hydroxy acid is about half as effective as the amino acid. Since, however, this estimate is derived from a comparison of only two experiments, each carried out with one small group of rats, only the qualitative implications of these results can be discussed.

It is known that the stock diet (5) adequately supplies the amino acid requirements even of growing rats, so that the adult animals used in this experiment must have been abundantly supplied with phenylalanine and tyrosine, both of which are present in considerable quantity in the casein of the diet (6).

The utilization of phenyllactic acid thus cannot be explained by a nutritional deficiency of phenylated amino acids; *i.e.*, of the compounds into which the hydroxy acid was converted. It follows that although the starting material is a substance foreign to the diet, and whatever the pathway of the conversion, this reaction must also be considered an *automatic process*.

If the hydroxy acid is not a constituent of the body tissues, nor even a short lived metabolic intermediate, the over-all conversion reaction cannot be viewed as part of the interrelated *normal* metabolic processes. However, phenyllactic acid is readily converted by oxidation to phenylpyruvic acid, which is regarded as a normal intermediate in the amination and deamination of phenylalanine. The results of this experiment might therefore be inter-

[2] This conversion requires amination in the α position and introduction of a hydroxyl group into the phenyl ring, but the experiments give no information as to the sequence of these metabolic steps; *i.e.*, the intermediates might be phenylpyruvic acid and phenylalanine or *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylpyruvic acid.

preted by assuming that the oxidation is a non-specific, automatic process; that if the organism has the ability to oxidize phenyllactic acid in the α position, the reaction will always take place when the hydroxy acid is present, irrespective of the composition of the diet and the nutritional state of the organism. The hydroxy acid is then introduced into what is probably a continuous cycle of reactions which must involve the formation of tyrosine.[3]

<div align="center">EXPERIMENTAL</div>

<div align="center">

Preparation of Deutero dl-Phenyllactic Acid

</div>

The two optically active β-phenyllactic acids have been prepared from the corresponding phenylalanines by the action of barium nitrite and sulfuric acid (7) and of sodium nitrite and sulfuric acid (8).

Deamination of *dl*-phenylalanine with sodium nitrite and sulfuric acid gave a product crystallizing from carbon tetrachloride in colorless needles, m. p. 92–93° (corrected), in a yield of 40 per cent. The melting point of this material was raised to 95–96°, with very little loss, by a second crystallization from carbon tetrachloride, chloroform, or benzene. When the deamination was carried out by shaking the amino acid in hydrochloric acid solution with the theoretical quantity of silver nitrite (9), a product which melted at 95–96° after only one crystallization was obtained in a yield of 55 per cent. The substance, m. p. 95–96°, gave theoretical C and H analysis figures for phenyllactic acid, but the melting point could be raised to 96–97° by one crystallization from water. Numerous subsequent crystallizations from water, benzene, and chloroform, however, did not further elevate the melting point. Erlenmeyer (10) gives a melting point of 98° (uncorrected) for β-phenyllactic acid.

6.5 gm. (0.04 mole) of deutero *dl*-phenylalanine containing 16.8 atom per cent deuterium were dissolved in 100 ml. of water containing 5.3 gm. (0.052 mole) of concentrated hydrochloric

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[3] A similar explanation would hold for the introduction of the phenolic group and the subsequent oxidation of the α-hydroxyl group if the conversion takes place through *p*-hydroxyphenyllactic and *p*-hydroxyphenylpyruvic acids.

acid, and the solution cooled to 0°. 8.0 gm. (0.052 mole) of finely powdered, freshly precipitated silver nitrite were then added and the suspension shaken at room temperature for 5 hours in a long necked, open flask containing glass beads. The reaction mixture was filtered, the silver chloride washed with water, and the combined filtrate and washings acidified to Congo red with sulfuric acid. The clear solution was extracted with five portions of ether, the combined extracts dried with calcium sulfate, and the solvent distilled off. The residual, almost colorless oil was dissolved in 80 ml. of carbon tetrachloride and the solution cooled to 0°. After filtration and washing with carbon tetrachloride the crystalline mass showed a slight tinge of greenish yellow color but melted at 96-97° (corrected). It was dissolved in 12 ml. of water and treated with a small amount of norit. At 0° this solution rapidly deposited 2.5 gm. of colorless needles which also melted at 96-97° (corrected). The carbon tetrachloride and aqueous mother liquors yielded a further quantity of material which, after two crystallizations from carbon tetrachloride and one from water, weighed 0.75 gm. and melted at 96-97° (corrected). These two fractions were combined and used for the feeding experiment.

The substance contained 18.5 ± 0.18 atom per cent deuterium.

Analysis—Calculated for phenyllactic acid containing 18.5 per cent deuterium, C 64.3, H 5.0; found, C 64.5, 64.4, H 5.0, 4.9.

The combined carbon tetrachloride mother liquors contained 1.8 gm. of a deep yellow uncrystallizable oil. It seemed possible that this oil was a mixture of lactones related to those formed by lactic acid and an alkaline hydrolysis was therefore attempted. The oil was dissolved in 1 M alcoholic sodium hydroxide and the solution boiled under a reflux for 5 hours. After a preliminary ether extraction of the hydrolysate, it was acidified and reextracted. The second ether extract yielded a yellow oil which in carbon tetrachloride solution deposited 1.0 gm. of very pale yellow crystals; m. p. 93-94°. A second crystallization removed the color and raised the melting point of this material to 95-96°: a mixed melting point with the main fraction showed no depression. Deutero *dl*-β-phenyllactic acid was thus obtained in a total yield of 64 per cent of the theory.

Biological Experiment

The deutero *dl*-phenyllactic acid was dissolved in 1 mole of sodium carbonate solution and added to the stock diet (5), the volume of the solution being so adjusted that the mixture could be worked up into a moderately stiff paste. The animals received 15 gm. of diet per rat per day, this quantity being completely consumed during a 24 hour period.

The procedures for the isolation, purification, and deuterium analysis of tyrosine were identical with those described for the previous experiments with deutero *dl*-phenylalanine (1).

SUMMARY

1. Deutero *dl*-β-phenyllactic acid was prepared from deutero *dl*-phenylalanine by the action of nitrous acid, and was added to a casein-containing stock diet of adult rats over a period of 10 days. Samples of tyrosine were isolated from the proteins of these animals and analyzed for deuterium.

2. The deuterium content of the tyrosine indicates that the amino acid is formed from phenyllactic acid despite the presence of abundant supplies of tyrosine and phenylalanine; it is suggested that the conversion is an automatic, non-specific process.

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A QUANTITATIVE REDUCTION-OXIDATION METHOD FOR THE ESTIMATION OF VITAMIN K₁ AND ASSOCIATED QUINONES AND NAPHTHOQUINONES

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Since the discovery and synthesis of vitamin K_1, interest in its biological rôle and that of the associated naphthoquinones and benzoquinones has been markedly stimulated (1). This situation has given rise to the need for a convenient and accurate method for their estimation. This paper describes a method whereby many quinone-like substances, whose standard oxidation-reduction potentials, E_0, are less than about 0.5 volt, may be assayed.

The method, in principle, consists of two stages, first catalytic reduction of the quinone to the hydroquinone and second the reoxidation of an aliquot part of the latter with a relatively stable and easily obtained dye, 2,6-dichlorophenol indophenol. This oxidative approach to the problem of quinone estimation has a very definite advantage over that usually used, *i.e.* titration with active reducing agents such as titanous chloride or sodium hyposulfite, in that the dye is stable against air oxidation. Thus none of the usual unwieldly protective devices is required. This lends to relatively easy manipulation and to the absence of the need for frequent restandardization. Furthermore the dye is its own indicator.

Aside from the fact that, as will be shown later, by the present method a wide variety of quinones may be successfully determined, it also seems to possess a reasonable degree of generality inasmuch as it has been found possible to assay vitamin K_1 in the presence of several natural oils such as castor, peanut, and sesame. Through the use of dyes of higher or lower standard oxidation-reduction potentials the range of titratable substances may be broadened

or narrowed at will and some degree of selectivity thus introduced. Such latitude may prove of value in special cases.

Table I contains a summary of the standard potentials of some of the substances dealt with in this communication. Values are given for pH 0 and 7. These will be of value in connection with the subsequent treatment.

TABLE I

Standard Oxidation-Reduction Potentials of Some Quinones, Naphthoquinones, and Dyes

Substance	Standard potentials at pH 0	Potentials at pH 7	Bibliographic reference No.
	mv.	mv.	
2,6-Dichlorophenol indophenol......	+648	+203	2
Tetrahydro-1,4-naphthoquinone.....	+614	+201*	3
Quinone...........................		+316*	3
α-Tocopherol......................		+200 (Apparent)	4
1,2-Naphthoquinone................	+576	+163	5
Methylene blue....................	+532	+1.5	6
1,4-Naphthoquinone................	+484	+71*	5
Duroquinone.......................	+466	+53*	7
2-Methyl-1,4-naphthoquinone.......	+408	−5*	5
2,6-Dimethyl-1,4-naphthoquinone...	+405	−8*	5
2-Methyl-3-phytyl-1,4-naphthoquinone (vitamin K_1)	+360	−53*	8
2,3-Dimethyl-1,4-naphthoquinone...	+340	−73*	5
Phthiocol.........................	+300	−113*	
Phenosafranine....................		−252	9

* Calculated assuming $E_n = E_0 + 0.059$ pH. This equation is valid when the acid dissociation constants of the phenolic groups of the reduced compound are small compared to the hydrogen ion concentration, a condition fulfilled for the hydroquinones and hydronaphthoquinones over the range of pH from 0 to 8.

EXPERIMENTAL

Dye—Considerable variation was observed in the behavior of samples of 2,6-dichlorophenol indophenol (sodium 2,6-dichlorobenzenoneindophenol) obtained from different sources. However, it has been found that the Eastman product behaves quite satisfactorily and is recommended for use in this assay. Since

2,6-dichlorophenol indophenol cannot be obtained in the pure state (it always contains some water and sodium chloride (2)), the proper amount to weigh out, in order to make a solution of about the desired strength, had to be determined empirically. To obtain a solution about 2.0 mM, it was found necessary to use 1 gm. of the solid dye per liter of solvent. After being shaken for about 15 to 20 minutes, the solution was filtered to remove undissolved solids, put in an amber bottle, and stored in a refrigerator when not in use. Under such conditions the dye solution retained its strength over periods of time up to 3 months. It was observed that some samples of the dye gave solutions having a violet-blue color; these were found to be valueless for purposes of titration and were rejected. Two criteria were found to be important in judging the value of a given sample of dye, first a clear green-blue color in the stock dye solution and second the absence of any red coloration at the end-point of the titration. With good dye *stable* end-points were always obtained.

Solvent—Both absolute ethanol and *n*-butanol have been used as solvents for the dye. It was found that only solvents of the best grade may be used in this work. Reagent grade *n*-butanol was found satisfactory but certain commercial grades of solvents proved valueless owing to the presence of small amounts of strong oxidizing agents (probably peroxides) which gave rise to unstable end-point characteristics. When titrations are to be run upon samples of natural oils, *n*-butanol must be used, since such oils are too insoluble in ethanol. For dissolving the specimen to be assayed 95 volume per cent *n*-butanol, containing 0.5 to 1 mg. of phenosafranine (Eastman) per liter, was used. The water is added for electrochemical reasons and the phenosafranine as a reduction indicator whose purpose will be apparent later.

Preparation of Raney's Nickel Catalyst—Raney's nickel catalyst was prepared in the usual manner (10), except that considerable care was given to the washing procedure to insure the removal of all traces of free alkali (both phenosafranine and vitamin K_1 are destroyed by the latter). The final washing was carried out with 95 per cent ethanol containing some acetic acid. A batch of catalyst may be considered satisfactorily washed when the wash alcohol from it, after standing, gives a neutral reaction with brom-thymol blue indicator. Finally all "fines" must be removed.

This was accomplished by shaking the prepared nickel catalyst with a relatively large volume of 95 per cent ethanol, allowing to settle for 1 to 2 minutes, and then quickly decanting off the liquid. This process was repeated a sufficient number of times until the residual catalyst settled off rapidly, leaving no appreciable cloud in the liquid phase. Final grading was made by
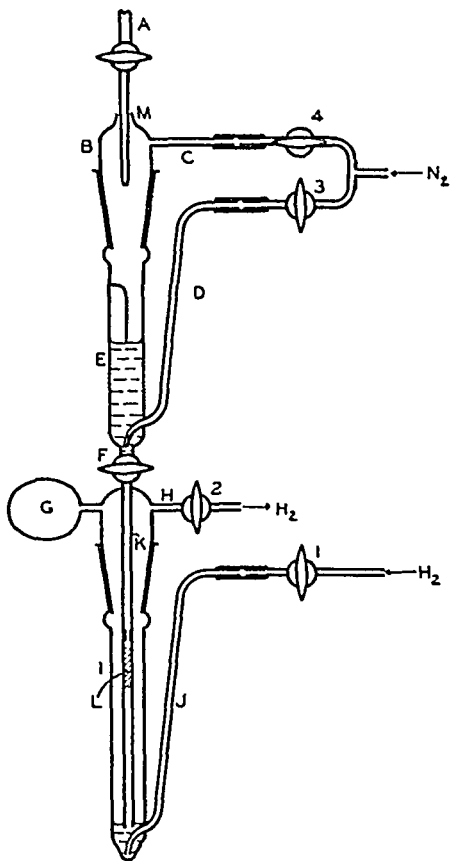
FIG. 1. Reduction-oxidation titration apparatus

washing the solid on a 400 mesh sieve with a stream of alcohol. The material remaining on the screen after this treatment was washed into a 15 cc. centrifuge tube and stored under alcohol ready for use. Such material is readily filtered through a cotton plug, giving a clear filtrate.

Apparatus—A cross-sectional sketch of the apparatus is presented in Fig. 1. It consists essentially of two chambers, E and I,

in the uppermost of which the actual titration is carried out, while in the lower the preliminary catalytic reduction is accomplished. The conical bottom of the lower chamber should be carefully fashioned so that the gas bubbles from jet J will continually sweep the catalyst into suspension. The volumes, to the ground joints, are about 30 cc. for the lower and 20 cc. for the upper chamber. The major diameters are about 22 mm. G is a rubber bulb of about 30 cc. capacity. The internal tube K is 5 mm. outside diameter and is provided with a constriction about half way up its length. The latter prevents the cotton plug L from being displaced into the upper chamber. Chamber E contains a glass pointer centrally located so as to define a volume of about 8 cc. The outlet M on head B is formed to fit the burette tip as illustrated. Burette A has a total volume of 5 cc. subdivided in hundredths. A stiff wire hook, inserted through the lower end of tube K, serves to insert and remove the cotton plug.

Gas Trains—Commercial tank nitrogen and electrolytic hydrogen were used. The hydrogen was conducted to jet J through a spiral gas-washing tower containing a quantity of the same stock solvent as that used in preparing the samples for assay. In this manner, the hydrogen was saturated with solvent vapor before entering chamber I and any change of concentration in I during reduction was avoided. A water seal blow-off located immediately adjacent to the tanks was provided in each train to avoid excessive pressures. Commercial tank nitrogen usually contains some oxygen, and since only 0.2 cc. of oxygen would be needed to reoxidize all the dihydronaphthoquinone contained in the aliquot taken from the average titration, a spiral gas-washing tower containing alkaline sodium hyposulfite solution (11) was included in the train. Care must be taken to see that the sodium hyposulfite used is fresh, as old samples were found to evolve hydrogen sulfide which is an active reducing agent for the quinones. Passage through a copper-filled tube at 500° would probably be a better procedure.

Procedure

The whole apparatus was thoroughly cleaned (final rinse with acetone), dried on a vacuum line, the cotton plug inserted into K as illustrated, and the apparatus set up on a stand. A small

cork was inserted in M and nitrogen gas allowed to flush out chamber E by entry at C and exit at the end of tube K. In the meantime, the lower chamber I was put into a wooden block stand, the H_2 gas started through tube J (at several bubbles per second), 10 to 12 cc. of the solution containing a weighed amount of the sample to be assayed pipetted into it, about 10 to 15 mg. of powdered potassium acetate added (as a buffer to keep the medium slightly alkaline), and finally a small amount (about the size of a pea) of the prepared nickel catalyst. The catalyst was always centrifuged in its storage tube and all free solvent drained off before each analysis in order to avoid dilution of the quinone solution. It is quite difficult to describe adequately the proper amount of catalyst to be taken for use, as only experience will suffice to give the operator a feel for the necessary quantity. However, the amount used should be sufficient to complete reduction in about 15 to 20 minutes.

Chamber I having been put in place with stop-cock 2 open, stop-cock F was closed, the stopper in M removed, the burette put in place at M, stop-cock 4 closed and 3 opened, and both gas streams allowed to continue. As reduction proceeds, the yellowish tinge, due to the quinone, of the reducing solution slowly disappears and the pink of the phenosafranine intensifies until all the quinone has been reduced; then the pink quickly disappears and reduction is completed. In practice reduction was continued about 5 minutes beyond the time necessary for the pink to disappear. During the reduction period the rubber bulb G was squeezed periodically to expel any air present within it. The completely reduced system is colorless. Stop-cocks 1 and 2 were then closed and the catalyst given several moments to settle. Stop-cock 3 having been closed and 4 opened, stop-cock F was opened and an aliquot part of the liquid blown up through the cotton plug into chamber E by means of the rubber bulb G. Transfer was continued until the surface of the rising liquid just touched the tip of the glass point. This was accurately adjusted with the aid of a small 7 × magnifier. Observation of the approach of the real and virtual images of the glass point aided in determining the point of contact. Stop-cock F was then immediately closed.[1] Stop-cock 4 was then closed and 3 opened. The

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[1] The volume in the upper chamber E defined by the glass point and the closed stop-cock F had been previously calibrated.

gas flow during the titration served two purposes, one to stir the solution and the other to exclude entry of air. Leucophenosafranine is very sensitive to gaseous oxygen and consequently should traces of the latter get into the system at any time during the foregoing manipulation, or as a result of inadequate flushing, the operator is promptly warned of that fact by the appearance of a pink coloration in the liquid in the upper chamber. Dihydrovitamin K_1 is very sensitive to air.

Titration with the 2,6-dichlorophenol indophenol dye was carried out at once. Initially the dye may be run in quite rapidly as the reaction rate is very fast.[2] Toward the end dropwise addition was resorted to. The addition of the first drop of dye brings up the pink of the phenosafranine, while the last drop of dye converts the pink-yellow color of the solution to a very distinct greenish blue. This color should be stable for not less than 5 minutes. If a red-violet color appears as the end-point, the result should be suspected, as it has been found that such a color is an indication of deterioration of the indophenol dye solution and fresh dye should be made. When butanol solutions are used, ample time should be allowed for the burette to drain before a reading is made. Calculation of the results are made in the obvious manner, the indophenol dye solution being expressed in terms of mg. per cc. of the standard substance.

DISCUSSION

From the data in Table I it will be noted that the potential of phenosafranine lies well below that of any of the known quinones or naphthoquinones and that fact makes possible its use as a reduction indicator. Clearly the disappearance of its pink color is a definite indication that the potential of the reducing system is below -252 millivolts (at pH 7), a simultaneous indication that any quinone or naphthoquinone present must have been reduced completely. Inasmuch as no control is easily exercised over the amount of catalyst taken for the reduction, the times of reduction are necessarily somewhat variable and the presence of such an indicator is a distinct aid in insuring completeness of the reduction stage during assays.

[2] This results, in part, from the slightly alkaline condition of the medium. On the acidic side the rate of reaction is much slower.

Tables II and III contain summaries of typical results. All the substances used were highly purified and carefully characterized by ultimate analyses and melting points. From the results in Table II it will be noted that a reproducibility of better than 1 per cent was attained. Those in Table III are of interest because they indicate an apparently anomalous behavior. As a rough

TABLE II

Reproducibility of Titrations

Substance	Apparent strength of dye
	mM
Vitamin K$_1$.............................	5.74, 5.67, 5.72
2,3-Dimethyl-1,4-naphthoquinone........	5.74, 5.76, 5.73, 5.71
2-Methyl-1,4-naphthoquinone...........	5.23, 5.25, 5.25

TABLE III

Summary of Anomalous Behavior of 2,6-Dichlorophenol Indophenol Titrations

Substance	Potential (pH 7)	Apparent strength
	mv.	*mM*
Phthiocol.................................	−113	6.21
Vitamin K$_1$................................	−53	5.72
2,3-Dimethyl-1,4-naphthoquinone..............	−73	5.72
2-Methyl-1,4-naphthoquinone	−5	5.25
Tocoquinone..............................	+50*	5.28
Durohydroquinone.........................	+53	5.23
2,5,6-Trimethyl-3-phytylbenzoquinone........	+50*	5.28

* The potentials of these quinones have never been measured but they must be within a few millivolts of that of durohydroquinone.

approximation it would seem that the apparent millimolar strength of the indophenol dye solution is some function of the potential of the substance being titrated. The explanation for this behavior is obscure but it was observed to persist even when quinhydrone was used as the oxidizing agent as well as when platinium oxide was used as the reduction catalyst. Samples of 2,6-dichlorophenol dye from different sources were found to give the same result. The existence of such an anomaly puts a definite

restriction on the choice of a substance for dye standardization. Thus if vitamin K_1 assays are to be undertaken, one must choose 2,3-dimethylnaphthoquinone as standard. Of course vitamin K_1 itself may be used if a sample of known purity is available. Since, in general, solids are much more readily purified than liquids, the former are preferable as standards.

In carrying out vitamin K_1 assays it was found that the best concentration range is about 0.5 to 1 mg. per cc. and the best dye strength about 4 to 5 mM. 1 per cent accuracy is attainable in this range. Lower concentrations may be used with a proportional loss of accuracy. It has been estimated that a total sample of about 0.1 mg. of vitamin K_1 constitutes the lower practical limit of this method unless changes are made in the dimensions of the apparatus so as to reduce its physical size and an attempt be made so to design the upper titrating chamber that advantage may be taken of observation through a greater depth of liquid. A factor of at least 6 could be obtained by such changes and the total required sample thus reduced to about 20 γ.

In the course of this work the possibility of using hydrogen sulfide or sodium hyposulfite as the reducing agent to replace the catalytic reduction was tried. Both substances actively reduce the naphthoquinones. However, the oxidation products of these reducing agents, namely sulfur and sodium sulfite, were found to be subject to further oxidation by 2,6-dichlorophenol indophenol with the result that unstable end-points were obtained. Clark and coworkers reported a similar experience during their investigations of the oxidation-reduction potentials of the substituted indophenols.

In addition to the substituted quinones and naphthoquinones listed in Tables I to III naphthotocoquinone also has been assayed successfully by this method. It behaved like duroquinone, as would be expected from its structure. Titration of 5,6,7,8-tetrahydro-2-methylnaphthohydroquinone gave clear evidence of an equilibrium in accord with the expectation to be derived from its potential relative to that of 2,6-dichlorophenol indophenol (Table I). It was not found possible to titrate either vitamin K_1 oxide or 2-methylnaphthoquinone oxide. The results seem to indicate that a catalytic reduction occurs but that the product is not a simple naphthohydroquinone but some substance of high poten-

tial, possibly one whose benzenoid nucleus has been saturated. The results of the titration indicate the presence of an equilibrium. In addition the oxidation product of the 2-methylnaphthoquinone oxide is a red colored compound suggestive of a hydroxy-substituted quinone.

As would be expected, 2-methyl-2-phytyl-3-dihydro-1,4-naphthoquinone does not titrate under the conditions of this method.

As was mentioned earlier in this paper, the method has been successfully applied to the assay of vitamin K$_1$ samples containing vegetable oils. These tests were carried out on synthetic samples of castor, sesame, and peanut oils to which known amounts of pure vitamin K$_1$ had been added. It was found necessary to dilute the oils about 1 to 5 with 95 per cent n-butanol (containing phenosafranine) in order to avoid foaming and to reduce the viscosity of the medium during the reduction and titration. Excellent checks between the assays and the known amounts present were obtained. The rate of reduction in the presence of some of these oils was found to be slower than in their absence and in consequence somewhat larger amounts of the nickel catalyst were used and the reduction time extended. It was also observed with oils present that occasionally the pink of the phenosafranine failed to reappear on addition of the first drop of the indophenol dye, although its behavior during reduction appeared normal. However, none of these phenomena seriously affects the method and many satisfactory assays for vitamin K$_1$ preparations in natural oils have been made. The accuracy is somewhat lower, being ±2 per cent.

Since the ultimate objective for development of this assay was application to biological media and since vitamin K$_1$ is a fat-soluble substance, it was deemed necessary to investigate what interference could be expected from other fat-soluble vitamins such as A and E.

To this end determinations were carried out with pure vitamin E and a highly concentrated vitamin preparation in cod liver oil alleged to contain 50,400 U.S.P. units per cc. of vitamin A and 5040 U.S.P. units per cc. of vitamin D. Perfect blanks were obtained in both cases, so that it appears that these substances cause no interference. It should be noted, however, that tocoquinone, a common oxidation product of vitamin E (4, 12) does titrate by

this method and hence caution should be taken when this method is applied to old, exposed preparations containing significant amounts of vitamin E. It is also probable that reducing sugars will cause interference, since 2,6-dichlorophenol indophenol has long been known to react with them. These are easily separated from vitamin K_1, however, by simple extraction methods.

The authors wish to express their sincere thanks to Dr. T. J. Webb for his frequent advice and criticism throughout the course of this work and to Dr. M. Tishler for his kindness in providing many of the substances used in it.

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THE ACTION OF CRYSTALLINE RIBONUCLEASE ON RIBONUCLEIC ACID

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The chemical changes in the molecule of ribonucleic acid occasioned by a thermostable enzyme of the pancreas, as first recorded by Jones (1) in 1920, have received recent attention by Dubos and Thompson (2), Schmidt and Levene (3), and Kunitz (4). However, in order that a definite rôle may be assigned to the enzyme, it appears that information other than that which is now at hand is required.

A perusal of the facts that are known concerning the structure of ribonucleic acid indicates that any enzymic action upon this substance should be reflected in the behavior of the constituent phosphate groups toward direct titration. The validity of this observation and its bearing on certain aspects of the structure of ribonucleic acid are shown in the following experiments.

EXPERIMENTAL

Commercial ribonucleic acid from yeast (Eastman or Merck) was purified according to the accepted procedures. Each of the preparations was analyzed for total nitrogen, phosphorus, sodium, and chlorine. Sodium chloride is a very difficultly removable contaminant of nucleic acid. Finally, the number of equivalents of acid per mole was measured for each preparation by the use of a glass electrode-electrometric titration apparatus. The glass electrode-electrometric titration apparatus was designed by Goyan, Barnes, and Hind (5).[1]

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[1] We wish to express our appreciation to Dr. F. M. Goyan who very kindly placed his own apparatus at our disposal.

Ribonuclease was isolated and obtained in a crystalline state as described by Kunitz (4). Aqueous solutions containing 1 mg. of enzyme per cc. were employed in the experiments.

The plan of the experiments was to observe the increase in equivalents of acid per mole that is effected by the enzymic action.
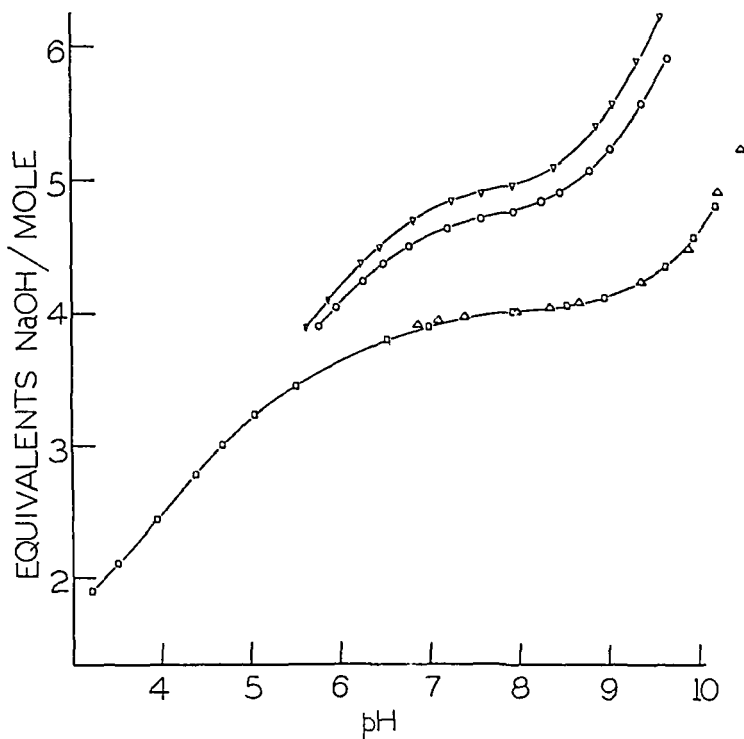
FIG. 1. Titration curve of ribonucleic acid, and of ribonucleic acid subjected to the action of ribonuclease. □ represents ribonucleic acid, △ ribonucleic acid incubated for 4 hours at 60° and pH 6.85 without the addition of ribonuclease, ○ ribonucleic acid incubated for 4 hours at 60° and an initial pH of 6.85 in the presence of ribonuclease, ▽ ribonuclease incubated for 7 hours at 60° and an initial pH of 6.85 in the presence of ribonuclease.

The following data, that are presented graphically in Fig. 1, will serve to illustrate the plan and procedure of the experiments. 4.49×10^{-4} mole of ribonucleic acid, previously dried to constant weight *in vacuo* over sulfuric acid, was transferred quantitatively into the titration compartment of the titrimetric apparatus with

the aid of a measured quantity of a solution of sodium hydroxide. This preparation of ribonucleic acid contained 15.40 per cent nitrogen, 9.55 per cent phosphorus, 0.24 per cent sodium, and 0.085 per cent chlorine. 0.055 per cent of the sodium is present in the form of NaCl. The remainder of the sodium (0.185 per cent) is assumed to be present in combination with the nucleic acid. The value for the combined sodium, expressed in equivalents, has been included in all the titrimetric data that are given later. The value for the molecular weight of ribonucleic acid is taken as 1286 (6). 17.96×10^{-4} equivalent of sodium hydroxide was required to titrate to pH 7.9, the point of inflection of the curve. Calculations from these data show that ribonucleic acid has four acidic groups in the range of the first and second dissociable groups of phosphoric acid.

In the experiments that were designed to illustrate the mode of action of the enzyme, a quantity of the preparation of ribonucleic acid, mentioned in the preceding paragraph, was weighed out, dissolved, and brought to pH 6.8 by the addition of a solution of sodium hydroxide of known equivalents as indicated by the titration curve. The solution was then diluted to a known volume by the addition of carbon dioxide-free distilled water. Ribonucleic acid and sodium ribonucleate form a buffer, hence the addition of other buffers is neither needed nor desired for reasons of the experiment. Aliquots of this solution were then treated in the following manner. (a) To 10 cc., 2 cc. of water were added. A 10 cc. aliquot, containing 3.02×10^{-4} mole, was titrated immediately. The pH was 6.85. 12.08×10^{-4} equivalent of sodium hydroxide was required to titrate to pH 7.9. This experiment served as a control to check both the initial pH and the original titration curve. (b) To 10 cc., 2 cc. of water were added. This was placed in a 60° water bath. At the end of 4 hours, a 10 cc. aliquot, containing 3.02×10^{-4} mole, was withdrawn and titrated. The pH was 6.85. 12.08×10^{-4} equivalent of sodium hydroxide was required to titrate to pH 7.9. This experiment shows that ribonucleic acid is stable at this pH and temperature. (c) To 10 cc., 1 cc. of water and 1 cc. of a solution of the enzyme were added. This was placed in a 60° water bath. At the end of 4 hours, a 10 cc. aliquot, containing 3.02×10^{-4} mole, was withdrawn and titrated. The pH, owing to the liberation of acidic

groups, had decreased to 5.76. 14.35×10^{-4} equivalent of sodium hydroxide was required to titrate to pH 7.9.   Calculations show the aliquot to contain 4.75 equivalents of acid per mole of ribonucleic acid.   Thus, in 4 hours, the enzyme liberated 0.75 equivalent of acid per mole.   (d) To 10 cc., 1 cc. of a solution of enzyme was added.   This was placed in a 60° water bath.   At the end of 4 hours, a second 1 cc. of a solution of enzyme was added.   At the end of 7 hours (total time), a 10 cc. aliquot containing 3.02×10^{-4} mole was withdrawn and titrated.   The pH had decreased to 5.61. 14.96×10^{-4} equivalent of sodium hydroxide was required to titrate to pH 7.9.   Calculations show the aliquot to contain 4.95 equivalents of acid per mole.   This corresponds to the liberation of 0.95 equivalent of acid per mole by the action of the enzyme.

Seven preparations of ribonucleic acid, other than the one already illustrated, were studied in similarly planned experiments.   Results similar to those given in the foregoing pages were obtained.

DISCUSSION

The number of equivalents of acid displayed per mole of ribonucleic acid in the range of the dissociable phosphoric acid groups is dependent upon the following conditions: (a) the mode of union of the individual mononucleotides in the simple tetranucleotide molecule, and (b) the mode of union of the tetranucleotides in the formation of a polymerized molecule as suggested by Schmidt and Levene (3).

An examination of the data that are published concerning the equivalents of acid of ribonucleic acid shows a lack of agreement.   Levene and Simms (7) claim that ribonucleic acid exhibits four primary phosphoric acid dissociations and one secondary phosphoric acid dissociation in an open chain structure.   They estimated that the dissociation constants of the four primary phosphoric acid groups as arranged in this structure should be roughly $-0.2, 0.4, 1.4,$ and 2.0.   The titration curve obtained in the present investigation indicates by its shape in the regions pH 4.7 to 7.9 that the anions of three of the phosphate radicals depress the ionization of the remaining acidic group to a greater extent than was estimated by these authors.

Makino (8), as well as Gulland and coworkers (9),[2] has not been

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[2] Cited by Gulland as unpublished data of Baker, Gulland, and Prideaux.

able to confirm the data of Levene and Simms, and holds that ribonucleic acid exhibits four primary phosphoric acid dissociations and no secondary phosphoric acid dissociation. These data are offered as support for the cyclic structure of ribonucleic acid, as proposed by Takahashi (10). The data of the present investigation confirm the data of Makino and of Gulland and coworkers.

The liberation of a fifth acidic group, by the action of the enzyme on ribonucleic acid as observed in the present investigation, may denote an opening of a cyclic structure, such as proposed by Takahashi, to form an open chain structure analogous to that proposed by Levene and Simms. The value of 5 equivalents per mole, taken together with the shape of the curve in the region of pH 6.0, indicates that the fifth acidic group is a secondary phosphate. Owing to the weakness of the fourth acidic group, as well as to the presence of two aromatic hydroxyl groups in the molecule, the points of inflection of both curves are at approximately the same pH.

The possibility that the enzyme may have the rôle of a depolymerase, as suggested by Schmidt and Levene (3), is not definitely excluded. The extent to which ribonucleic acid may occur in a polymerized state is not clear; in fact the experimental data heretofore published (11) do not indicate a high degree of polymerization, if any.

In contrast to ribonucleic acid, it must be emphasized that there is ample evidence to show that desoxyribonucleic acid may exist in a highly polymerized state (12–14).

The data of the present investigation may be examined to ascertain their support in assigning the rôle of the enzyme to that of a depolymerase. It is clear from the number of acid equivalents per mole that the phenomenon of polymerization must involve the loss of water between the secondary hydroxyl of a phosphate group and any of the following reactive groups: (a) the hydroxyl group of guanine, or of uracil; (b) the amino group of adenine, guanine, or cytosine; (c) the primary alcoholic group of position 5, or the secondary alcoholic group of position 2 of the ribose; (d) the primary hydroxyl group of another phosphate group.

In the event of such polymerizations, the number of equivalents of acid per mole should approach 4 as the number of tetranucleo-

tides in the polymer increase. A depolymerase would catalyze the hydrolysis of the secondary phosphate linkages and effect the liberation of a fifth acidic group.

In conclusion, it has been shown that the preparations of ribonucleic acid employed in this investigation have 4 equivalents of acid per mole in the regions of the dissociable phosphoric acid groups. These data are compatible with either (a) the cyclic structure proposed by Takahashi, or (b) the open chain structure proposed by Levene and Simms, provided a highly polymerized molecule is assumed wherein the secondary hydroxyl of a phosphate group is involved in the polymerization. The action of ribonuclease effects the liberation of a fifth acidic group. This action may be regarded either as (a) an opening of the cyclic structure, or (b) depolymerization. At the present time, there is little direct evidence to support polymerization in ribonucleic acid. No information concerning the type of linkages that are hydrolyzed by the enzyme can be offered. Further work is in progress.

SUMMARY

1. The titration curves of eight different preparations of ribonucleic acid have been studied.

2. In contradiction of Levene and Simms (7), and in agreement with Makino (8) and Gulland and coworkers (9), the number of equivalents of acid within the range of the dissociable phosphoric acid groups have been found to be 4 per mole.

3. In confirmation of a recent report by Kunitz (4), the thermostable enzyme of the pancreas has been isolated in a crystalline state.

4. The action of the enzyme upon ribonucleic acid has been studied by observing the increase in titratable acidity effected in solutions of the buffer pair, ribonucleic acid-sodium ribonucleate. The increase in equivalents of acid is found to approach 1 as a limiting value. Titration data place the liberated acidic group in the range of a secondary phosphoric acid dissociation.

5. The bearing of these data on the open chain structural formula proposed by Levene and Simms (7), the cyclic structure proposed by Takahashi (10), and on the polymerization of tetranucleotides is discussed.

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THE ACID STRENGTH OF BILE ACIDS

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A correct knowledge of the strength of the bile acids is important for certain physicochemical investigations of these acids and also for a correct interpretation of their action in the utilization of fats, fat-soluble vitamins, and drugs.

Of the previous work on the dissociation constants of some of these acids (1–5) that of Josephson is the most extensive. Sobotka ((6) p. 141) states, "Josephson's values deserve first consideration, should acid dissociation constants be required in the physicochemical investigation of other properties of pure bile acids."

Several considerations have led us to believe that a redetermination of these values is desirable. Although Josephson has made elaborate corrections for ionic strength, his electrometric determinations were made on supersaturated colloidal solutions, which casts some doubt on the meaning of his constants. We would expect the bile acids studied to have dissociation constants about equal to the higher saturated aliphatic acids, with values in the range of 1.0 to 1.4×10^{-5}. These bile acids can be considered as derivatives of valeric acid with the sterol nucleus substituted in the γ position. The unsubstituted sterol nucleus would be expected to have an acid-weakening effect. The hydroxyl and ketonic groups substituted on the sterol nucleus have an acid-strengthening effect but they are so far removed from the carboxyl group that it can be predicted that their effect would be small. The net result would be an acid of about the same strength as n-caproic acid. On the other hand, Josephson's value for desoxycholic acid, $K_a = 3.8 \times 10^{-7}$, is only one-thirty-fifth as large as the K_a value (1.32×10^{-5}) for n-caproic acid.

Since the only difference in structure between cholic and desoxycholic acids is a hydroxyl group in the 7 position, which is 8 carbon atoms removed from the carboxyl group, one would expect these acids to have almost identical K_a values. Josephson's K_a value for cholic acid (64.6×10^{-7}) is 17 times as great as his value (3.8×10^{-7}) for desoxycholic acid.

Lithocholic acid also would be expected to have a K_a value almost identical with desoxycholic and cholic acids. A current opinion, however, is that lithocholic acid is considerably weaker than the other bile acids and that this difference in acidity is the reason that lithocholic acid can be separated from the other acids by fractional precipitation with mineral acid ((6) pp. 78, 141, (7)). Our evidence indicates that this interpretation is incorrect and that the difference in acidity is entirely too small to account for the observed separation. It most probably comes about as a result of the marked difference in solubility between lithocholic and other bile acids.

<div align="center">EXPERIMENTAL</div>

The measurements were carried out in 50 and 66.6 per cent by volume alcohol-water solutions which gave no evidence of any colloidal material when examined with the ultramicroscope.

The pH of the solutions was determined with the glass electrode in connection with the universal pH meter and simplified vacuum tube electrometer of Goyan, Barnes, and Hind (8). The apparatus and primary standard were the same as those used by these authors. Temperatures were maintained at $25° \pm 0.2°$.

The dissociation constants were calculated assuming the relations

$$pH = -\log (H^+) \text{ and } K_a = \frac{(H^+)\left[(Na^+) + (H^+) - \frac{K_w}{(H^+)}\right]}{M - \left[(Na^+) + (H^+) - \frac{K_w}{(H^+)}\right]}$$

where M is the total concentration of undissociated acid and salt (9). The value of K_w used for the 50 per cent alcohol was 1.5×10^{-15}. A discussion of the significance of dissociation constants thus calculated has been given by Branch and coworkers (10, 11). An alcohol correction was made for the glass electrode (12).

Materials—Lithocholic acid, m.p. 187–188°; $[\alpha]_{5461}^{25} = +40.6°$; equivalent weight 378.

Desoxycholic acid, m.p. 174–175°; equivalent weight 391.

Apocholic acid, m.p. 172–173°; equivalent weight 392.

Cholic acid, m.p. 197–198°; equivalent weight 407.

Dehydrodesoxycholic acid, m.p. 185–186°; equivalent weight 387.

Dehydrocholic acid, m.p. 234–235°; equivalent weight 399.

n-Caproic acid, b.p. 96.4–96.6° at 8.5 mm.; equivalent weight 116.5.

Phenylacetic acid, m.p. 76–77°; equivalent weight 136.3.

Cinnamic acid, m.p. 133–134°; equivalent weight 148.0.

Benzoic acid, m.p. 121.5–122°; equivalent weight 121.8.

Results

The dissociation constants given in Tables I and II are directly comparable with those measured in the same solvent by other investigators using the same method (10). The variation of the pK_a values for the different per cent neutralization gives an estimate of their accuracy.

The difference between the strongest and the weakest of these six bile acids is only 0.09 pK_a unit and the strongest bile acid is only 0.02 pK_a unit weaker than *n*-caproic acid. Hence our prediction that these bile acids should have very nearly the same acid strength and that their strengths should be close to that of *n*-caproic acid is experimentally verified.

There is also a precise agreement between the experimentally determined strengths of these bile acids and their relative strengths as predicted from the known acid-strengthening character of the groups on the sterol nucleus. Thus ketone groups are more acid-strengthening than hydroxyl groups and hydroxyl groups are more acid-strengthening than double bonds; hence we would expect the following order of acid strength with respect to the groups substituted on the sterol nucleus: 3 ketone > 2 ketone > 3 hydroxyl > 2 hydroxyl, 1 double bond > 2 hydroxyl > 1 hydroxyl. This is precisely the order found; *viz.*, dehydrocholic > dehydrodesoxycholic > cholic > apocholic > desoxycholic > lithocholic.

Dissociation constants are smaller in alcohol-water than in pure

TABLE I

Dissociation Constants of Some Bile Acids and Caproic Acid in 50 Per Cent by Volume Alcohol-Water Solutions

Acid		Amount neutralized with NaOH	pH	pK$_a$	K$_a$, × 10^{-7}
	M	*per cent*			
Desoxycholic	0.01129	40	6.03	6.20 ⎫	
	0.01075	50	6.20	6.20 ⎬	6.33
	0.01133	60	6.37	6.19 ⎭	
Apocholic	0.01043	40	6.00	6.17 ⎫	
	0.00827	50	6.20	6.20 ⎬	6.40
	0.00755	60	6.39	6.21 ⎭	
Cholic	0.00468	40	5.99	6.17 ⎫	
	0.00448	50	6.19	6.19 ⎬	6.61
	0.00531	60	6.36	6.18 ⎭	
Dehydrodes-oxycholic	0.01002	40	5.96	6.14 ⎫	
	0.00982	50	6.15	6.15 ⎬	7.19
	0.01014	60	6.32	6.14 ⎭	
Dehydrocholic	0.00905	40	5.96	6.14 ⎫	
	0.00903	50	6.14	6.14 ⎬	7.30
	0.00958	60	6.31	6.13 ⎭	
Caproic	0.01266	40	5.92	6.10 ⎫	
	0.01171	50	6.12	6.12 ⎬	7.79
	0.01272	60	6.28	6.11 ⎭	

TABLE II

Dissociation Constants of Lithocholic and Desoxycholic Acids in 66.6 Per Cent by Volume Alcohol-Water Solutions

Acid		Amount neutralized with NaOH	pH	pK$_a$	K$_a$, × 10^{-7}
	M	*per cent*			
Lithocholic	0.00737	40	6.53	6.71 ⎫	
	0.00781	50	6.73	6.73 ⎬	1.86
	0.00627	60	6.93	6.75 ⎭	
Desoxycholic	0.00753	40	6.50	6.68 ⎫	
	0.00778	50	6.71	6.71 ⎬	1.95
	0.00694	60	6.91	6.73 ⎭	

water. The difference, however, is not constant for different acids; hence an exact calculation of K_a values in one solvent can-

not be made from those in another solvent. We can make an approximate calculation and whether we take an average of the ratios in Table III, 17.4:1, or whether we use the ratio for *n*-caproic acid, 17.0:1, makes little difference. The ratio 17.0:1 was used in calculating the values in Table IV.

The K_a of phenylacetic acid in water has been measured to get a direct comparison between constants determined by the apparatus and method we have used and the true thermodynamic constants.

TABLE III

Ratio of K_a Values in Water to K_a Values in 50 Per Cent Alcohol-Water

Acid	K_a water (13), $\times 10^{-5}$	K_a 50 per cent alcohol-water	$\dfrac{K_a \text{ water}}{K_a \text{ 50 per cent alcohol-water}}$
n-Caproic	1.32	7.79×10^{-7}	17.0
Phenylacetic.....	4.88	3.16×10^{-6}	15.4
Cinnamic	3.65	2.57×10^{-6}	14.4
Benzoic	6.27	2.75×10^{-6}	22.8

TABLE IV

Calculated Approximate K_a Values of Bile Acids in Water from K_a Values in Alcohol-Water Solutions

Acid	Groups substituted on sterol nucleus	K_a, calculated, $\times 10^{-5}$	K_c, Josephson's values
Lithocholic	1 hydroxyl	1.02	
Desoxycholic .	2 "	1.08	3.8×10^{-7}
Apocholic	2 " 1 double bond	1.09	
Cholic	3 hydroxyl	1.12	6.46×10^{-5}
Dehydrodesoxycholic	2 ketone	1.22	
Dehydrocholic	3 "	1.24	
Caproic		1.32	

We obtain a value of 5.15×10^{-5} in comparison with Dippy's thermodynamic value of 4.88×10^{-5} (13).

SUMMARY [1]

From theoretical considerations it is predicted that lithocholic, desoxycholic, apocholic, cholic, dehydrodesoxycholic, and dehydrocholic acids should have almost identical dissociation constants of about the same value as for n-caproic acid. Some of the accepted values in the literature appear to be in error, as they vary greatly and are much smaller than the value for n-caproic acid. The dissociation constants of these acids have been measured in alcohol-water solutions and the approximate values for these constants in water have been calculated.

The experimentally observed dissociation constants of these bile acids, both in regard to magnitude and relative strengths, are completely in accord with the predictions made on a basis of the acid-strengthening and acid-weakening groups present in the acids.

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THE PROSTHETIC GROUP OF SULFHEMOGLOBIN

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Treatment of hemoglobin with H_2S and O_2 gives rise to the formation of a green pigment, sulfhemoglobin. In contradistinction to the transformation of hemoglobin into HbO_2, HbCO, Meth-Hb, and other hemoglobin derivatives the transformation into sulfhemoglobin is not reversible. The nature of this reaction is as yet unknown (1). An attempt was made therefore to isolate the prosthetic group of sulfhemoglobin and to clear up its constitution.

Difficulties arose first of all in preparing pure sulfhemoglobin. The typical absorption band in the red region of the spectrum after having reached a certain intensity showed a gradual decrease of its extinction during prolonged treatment with H_2S and O_2 (Fig. 1). Probably the nascent sulfhemoglobin is autocatalytically destroyed later on by traces of H_2O_2, the formation of which from H_2S and O_2 has been proved (2, 3).

Contrary to hemoglobin and oxyhemoglobin, sulfhemoglobin is not split into a hemin and globin by the action of dilute acids. Neither boiling acetic acid and NaCl (Schalfejef) nor oxalic acid and acetone (4) cause the formation of hemin. We treated sulfhemoglobin therefore *with pepsin and HCl*, a method used first by von Zeynek (5) for the splitting of Hb. By repeated digestion with pepsin the bulk of the protein compound was split off. But contrary to the results with oxyhemoglobin *5 to 10 per cent of the globin adhered to the pigment.* Instead of pure hemin (6) a brown hemin-protein compound was obtained. It will be designated in

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the following as *sulfheminproteose*, in analogy to heminproteoses produced by the action of trypsin on hemoglobin (6). A similar sulfheminproteose was obtained by digestion of sulfhemoglobin with papain.
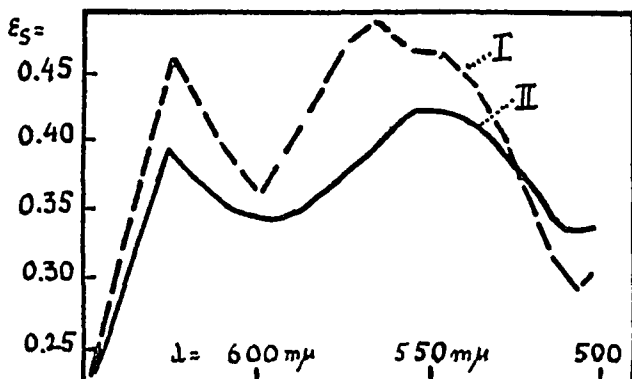
FIG. 1. Absorption curves of hemoglobin-sulfhemoglobin mixtures. Curve I = 10 hours treatment, Curve II = 20 hours treatment with SH_2 and O_2.
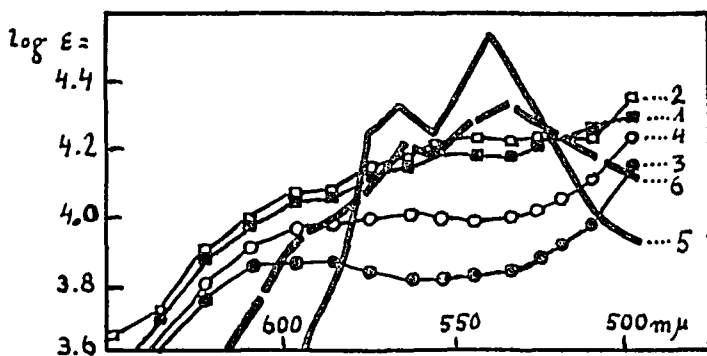
FIG. 2. Absorption curves of hemin (Curves 1, 3, 5) and of sulfheminproteose (Curves 2, 4, 6) in 1 per cent NaOH (Curves 1 and 2), after addition of NaCN (Curves 3 and 4) and after reduction with $Na_2S_2O_4$ (Curves 5 and 6).

Sulfhemoglobin contains 0.32 to 0.35 per cent of Fe, as does pure hemoglobin. This iron is not split off by treatment with pepsin-HCl. Its linkage to the pigment is just as strong as in hemin. The absorption spectrum of the sulfheminproteose is *a typical hemin spectrum* and resembles closely the spectrum of protohemin, both with regard to the position of the absorption bands and to

their intensity (Fig. 2). Just like hemin, the sulfheminproteose is converted into a bright red cyanide derivative by NaCN and into a typical ferro compound (hemochromogen) by $Na_2S_2O_4$. The sulfheminproteose contains 2 to 3 per cent of iron and 5 to 10 per cent of sulfur, corresponding approximately to 7 S atoms for each Fe atom. The bulk of this sulfur is adsorbed colloidal sulfur, which can be removed to some extent by dissolving the proteose in NaOH and reprecipitating with acetic acid, and to a greater extent by extraction with hot benzene.

From diffusion experiments with alkaline solutions of a sulf-hemiproteose containing 3 per cent of Fe a molecular weight of at least 19,000 results for sulfheminproteose. This corresponds to about 10 hemin molecules per molecule of sulfheminproteose. Apparently the sulfheminproteose just as hemin (7) is associated in its alkaline solutions.

Sulfheminproteose is insoluble in a saturated solution of HBr in glacial acetic acid and does not lose its iron, even after prolonged treatment with this reagent. The *cleavage of sulfheminproteose was achieved by the action of concentrated HCl at 100°*. Under these conditions the iron is split off, the protein component is hydrolyzed, and the bulk of the pigment is obtained as an insoluble precipitate which can be dissolved in dilute NaOH or in concentrated sulfuric acid. The absorption maxima of these solutions are as follows:

629 mμ, 576 mμ, 545 mμ, 509 mμ in N NaOH
603 " (593 "), 556 " in concentrated H_2SO_4

These are typical porphyrin absorption bands. But contrary to the behavior of most prophyrins the *precipitated porphyrin* is insoluble in dilute HCl and in organic solvents. Its elementary analysis corresponds to the formula $C_{34}H_{36}N_4O_8S_2$, differing from protoporphyrin, $C_{34}H_{34}N_4O_4$, by an excess of 2 S and 4 O atoms (perhaps 2 H atoms). Oxidative destruction of the porphyrin with HNO_3 (8) produces neither methylsulfonic acid nor other alcohol-soluble S compounds. No S-containing ether-soluble substances were obtained after oxidation of the porphyrin with chromic acid (9) nor after reductive cleavage with tin and HCl. We conclude from these experiments that *no sulfur-containing pyrroles* have been formed in these reactions. The *sulfur is not*

removed from sulfheminproteose after *heating with resorcinol* to 190° (Schumm (10)), but the absorption spectrum is shifted after this treatment 5 mμ towards the blue region. This corresponds perfectly with the analogous reaction of protohemin, whose absorption spectrum is shifted in the same direction after fusion with resorcinol, owing to a loss of the vinyl groups (Schumm). Apparently the S atoms of the insoluble porphyrin are not linked to its vinyl side chains.

Sulfoporphyrins with SO_3H groups have been described by Treibs (11). But no such groups could be detected in our porphyrin. Titration of the porphyrin with NaOH indicated the presence of only two acid groups, apparently the COOH groups of protoporphyrin. Methylation gave rise to two methoxy groups, most probably corresponding to a methylation of the two carboxyls.

We conclude from the failure to obtail sulfur-containing pyrrole derivatives, and from the result of the pyro reaction with resorcinol, that the S atoms are linked to the methene C atoms of the porphyrin, probably in the following way.
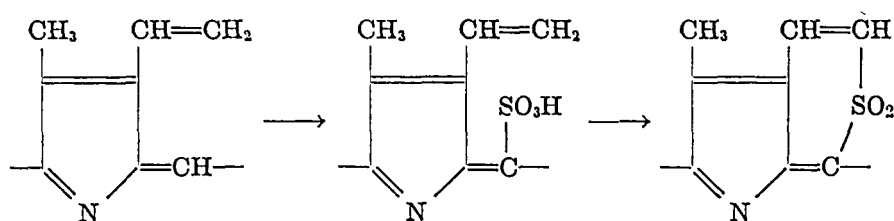
It must be emphasized that the above reaction of the vinyl groups has nothing to do with the appearance of the typical absorption bands of sulfhemoglobin, since a similar spectrum is obtained by the action of H_2S and oxygen on mesohemoglobin, in which the vinyl groups are replaced by ethyl groups.

The green color of sulfhemoglobin has led some authors to the opinion that sulfhemoglobin is related to the green bilirubinoid pigments (12, 13) with an open porphin ring. But contrary to the different green pigments described by Warburg (14), Edlbacher (15), Lemberg (16), and Barkan (17) and their coworkers sulfhemoglobin and sulfheminprotcose do not lose their iron after treatment with dilute HCl. Since sulfhemoglobin is different from the green pigments mentioned above, the question arises

whether the typical absorption spectrum of blood observed after treatment with phenacetin or with aniline derivatives is correlated to sulfhemoglobin or to one of the green pigments with labile iron.

With regard to the iron linkage only one of the green pigments resembles sulfhemoglobin; *viz.*, the green pigment produced by the action of KCN and H_2O_2 on Hb (Davis (18), Barkan and Schales (12)). Its iron is not split off by dilute HCl. It differs from sulfhemoglobin by its absorption band in the red region, which does not appear before addition of a reducing agent (12). In contradistinction to our statement Barkan and Schales (19) have found an increase of labile iron after treatment of Hb with a great excess of H_2S and O_2. Their results are confirmed below, but no significant increase of labile iron was found in preparing sulfhemoglobin from Hb according to our own method and during digestion with pepsin-HCl. We conclude therefore that the formation of labile iron has nothing to do with the formation of sulfhemoglobin and that it must be attributed to the secondary destruction of sulfhemoglobin by H_2O_2.

Sulfhemoglobin can be prepared from Hb under very mild conditions; *i.e.*, in cold neutral solution. The typical absorption spectrum of sulfhemoglobin appears almost instantaneously. Hence profound chemical reactions of the porphyrin nucleus can be excluded as a first cause of the altered spectrum. Michel (20) has proved recently in solutions of HbO_2 partially converted to sulfhemoglobin that the latter contains only 1 atom of extra sulfur per iron atom. Most likely the typical absorption spectrum of sulfhemoglobin develops as soon as one of the methene groups is substituted by a sulfur-containing group. The alteration of the spectrum may be due to a coordinating bond between iron and the S-containing group.

EXPERIMENTAL

Preparation of Sulfhemoglobin—Crystalline HbO_2 was prepared from horse blood corpuscles with the aid of toluene (21) or alcohol (22). A 20 per cent solution of crystalline Hb was perfused alternately with H_2S and O_2 or was slightly shaken in a mixture of both gases ($H_2S:O_2 = 2:1$). The progress of the reaction was controlled spectroscopically (1). After 10 hours the spectrum showed no further appreciable alteration. Now the precipitated sulfur

and denatured protein were filtered off and the absorption curve of the clear dark green solution Sulfhemoglobin I was determined (Fig. 1, Curve I). For that purpose 1 ml. of the solution was diluted with 99 ml. of 0.1 per cent sodium carbonate, reduced by a trace of $Na_2S_2O_4$, and measured in a Hüfner spectrophotometer. A part of Sulfhemoglobin I was treated again for 10 hours with the gas mixture (Sulfhemoglobin II) and the absorption measurement repeated (Fig. 1, Curve II). The protein concentration had fallen within these 10 hours from 17.5 to 15.5 per cent. The curves of Fig. 1 indicate the extinction coefficients of a 0.1 per cent protein solution.

Iron Determination—Iron was determined by the method of Kennedy (23). 1 ml. of 19.3 per cent SHb = 0.65 mg. of Fe = 0.35 per cent Fe. In 5 ml. of the same solution 0.022 mg. of labile iron was found by Barkan's (24) method, or less than 1 per cent of the total iron. Other preparations of sulfhemoglobin contained 0.32 to 0.35 per cent of Fe and 1 to 5 per cent of the total iron in the labile form.

Action of Acids on Sulfhemoglobin—The dark green solution of Sulfhemoglobin I was heated in the usual way with glacial acetic acid and NaCl. No hemin crystals were formed. The solution of sulfhemoglobin (20 per cent) gave a dark green precipitate after addition of acetone, but no pigment was extracted from this precipitate by a 4 per cent solution of oxalic acid in acetone. A solution of HbO_2 prepared from the same blood after addition of acetone gave a brown precipitate, the pigment of which was extracted almost quantitatively by the treatment with oxalic acid solution.

Treatment of SHb with Pepsin-HCl—480 ml. of a 10 per cent solution of Sulfhemoglobin I were adjusted to pH 1 to 2 by addition of HCl. 0.5 gm. of pepsin was added and the mixture kept at 38° for 2 days. The pigment was then precipitated, almost quantitatively, by neutralizing the free HCl with sodium acetate, with Congo paper as indicator. The precipitate was filtered off, washed with 0.1 per cent acetic acid, and treated again with pepsin and HCl. This was repeated two or three times until a negative biuret test was obtained in the clear filtrate. Now the sulfheminproteose was dissolved in warm 1 per cent NaOH, precipitated again with acetic acid, washed with water, dried at reduced

pressure, and extracted with boiling benzene in a Soxhlet apparatus. 6.036 gm. of a dark brown powder were obtained. It contained 2.2 per cent Fe, that is a total of 133 mg. of Fe, whereas the collected clear and slightly brownish filtrates of the repeated precipitations contained altogether 11.9 mg. of iron.

Action of Papain on SHb—500 ml. of 10 per cent sulfhemoglobin solution were kept at 38° with 50 ml. of 0.1 N phosphate buffer solution, pH 6.3, 1 ml. of toluene, and 0.25 gm. of papain. As for the rest, the treatment resembled the procedure with pepsin. After repeated action of papain 4.6 gm. of sulfheminproteose were isolated, containing 3.7 per cent of Fe and 8.8 per cent of S. Determinations of sulfur were performed according to Waelsch and Klepetar (25).

Properties of Sulfheminproteose—For spectrophotometric measurement a 0.0002 N solution of sulfheminproteose in 1 per cent NaOH was prepared from sulfheminproteose containing 2.4 per cent of Fe (100 ml. of this solution contained 1.12 mg. of iron). An equimolecular solution of pure protohemin in NaOH was prepared. Both solutions were measured in a Hüfner spectrophotometer (a) directly, (b) after addition of an equal volume of 10 per cent NaCN, and (c) after further addition of 30 mg. of $Na_2S_2O_4$ to 10 ml. of solution (b). Because of the slow reaction of sulfheminproteose with cyanide and hyposulfite in the cold, all solutions were kept 20 minutes in a boiling water bath. The result of the experiment is shown in Fig. 2.

200 mg. of sulfheminproteose were kept 15 minutes at 190° with 20 gm. of resorcinol. Then the resorcinol was dissolved in water and the pigment precipitated by addition of acetic acid and solid NaCl and reprecipitated from alkaline solution with acetic acid. The sulfur content decreased after fusion with resorcinol from 7.0 to 2.55 per cent, whereas the Fe content remained unchanged at 2.9 per cent. The maximum of the first hemochromogen band, after treatment with $Na_2S_2O_4$ in 0.01 N NaOH and addition of 5 per cent pyridine, was determined spectroscopically as follows:

Sulfheminproteose...552–553 mμ; after fusion with resorcinol.............................542 mμ
Protohemin.........559 mμ; after fusion with resorcinol..547 "

In eight different preparations of sulfheminproteose 2 to 4 per cent of iron and 7 to 10 per cent of sulfur were found. Most probably the percentage of iron depends on the integrity of the sulfhemoglobin, from which the sulfheminproteose has been prepared, and on its destruction by H_2O_2.

1.5 gm. of sulfheminproteose were dissolved in 150 ml. of 1 per cent NaOH and placed in the diffusion apparatus, No. 36b MG4 of Schott (Jena). The diffusion of the pigment in 1 per cent NaOH was measured colorimetrically. In the course of 7 days 3.85 per cent of the total pigment had passed the sintered glass membrane, corresponding to 0.55 per cent in 24 hours. The same experiment with 10 per cent sucrose solution brought about the diffusion of 4.1 per cent of the total sugar in 24 hours. Its concentration was determined by refractometry.

Preparation of a Porphyrin from Sulfhemoglobin—2.0 gm. of sulfheminproteose in a flask with a reflux condenser were digested with 100 ml. of 25 per cent HCl in the boiling water bath. After 8 hours the clear, slightly olive-colored solution was decanted from the insoluble pigment and replaced by fresh HCl. This was repeated once or twice until no ferric ions were detectable in the liquid. During the hydrolysis a considerable sublimate of crystalline sulfur accumulated in the reflux condenser. The insoluble brown pigment was centrifuged off and dissolved in warm diluted NaOH. It was precipitated then by acetic acid and washed with water, alcohol, ether, and hot benzene. Finally 0.609 gm. of dry pigment was obtained. Analysis of two different preparations gave the following results.

	C	H	N	S	Cl	O (calculated)
Porphyrin I	60.0	5.27	8.02	8.09	1.03	17.3
" II	60.4	5.18	7.54	7.65	0	16.8
$C_{34}H_{36}N_4O_2S_3$. Calculated	59.1	5.20	8.09	9.24	0	18.5

Nitrogen was determined by the Dumas method. Kjeldahl nitrogen determinations gave low values, which could be prevented by addition of potassium persulfate. The collected hydrolysis liquids, after centrifugation of the pigment, contained appreciable amounts of hydrogen sulfide, but neither sulfate nor sulfite.

A solution of 0.797 gm. of the porphyrin in 13.8 ml. of 0.25 N NaOH required 7.5 ml. of 0.1 N HCl for neutralization with

phenolphthalein as indicator. This corresponds to 2.35 equivalents of NaOH for each 4 N atoms (= one porphin ring). 0.1 gm. of the porphyrin was suspended in dry methanol, saturated with HCl gas, and evaporated at reduced pressure. In 31.1 mg. of the dry residue (15 mm., 100°) O-methyl was determined in the micro apparatus of Pregl. Titration of the methyl iodide according to Kirpal and Bühn (26) required 8.28 ml. of 0.01 N silver nitrate, corresponding to 1.9 methyl groups for each 4 N atoms. The methylated porphyrin was insoluble in pure methanol, but soluble after addition of a drop of concentrated HCl. Apparently the methylated product still contains basic groups, most probably the basic pyrrole nitrogen atoms of the original porphin ring.

Comparison of Sulfhemoglobin with Other Green Hb Derivatives— The green pigment of Edlbacher and von Segesser (15) was prepared according to these authors from horse blood corpuscles by treatment with ascorbic acid and oxygen. The green amyl alcoholic solution of the pigment lost its iron after shaking with N HCl. The amyl alcohol was evaporated at room temperature at reduced pressure over solid KOH and the green residue was dissolved in ether and drawn, for chromatography, through a column of pure talcum. Colorless substances (lipids) were extracted from the column by chloroform, whereas the green pigment was developed by ethyl alcohol. It was practically free of iron (1.0 mg. = 0.001 mg. of Fe).

Twice recrystallized hemoglobin from horse blood was transformed into a green pigment by treatment with KCN and H_2O_2 according to Barkan and Schales (12). *The solution was neutralized* by addition of oxalic acid and precipitated by addition of twice its volume of acetone. The precipitate was washed with acetone and then treated with a solution of 4 per cent oxalic acid in acetone. No pigment passed into the acid solution from the precipitate.

*Mesosulfhemoglobin—*Mesoporphyrin was transformed into mesohemin by the usual treatment with ferric chloride and sodium acetate in glacial acetic acid. Globin was prepared from ox blood according to Laporta (27). Slightly alkaline solutions of mesohemin and globin were coupled according to the procedure of Hill and Holden (28) and the solutions of mesohemoglobin then perfused with a mixture of H_2S and O_2. The spectrum of the alkaline

mesomethemoglobin with its two bands in the green region was replaced by the spectrum of mesosulfhemoglobin, with a characteristic sharp band in the red region at 617 mμ. The analogous band of sulfhemoglobin is situated at 626 mμ. But it is well known that all spectra of meso derivatives show a typical shift to the short wave region in comparison with proto derivatives.

SUMMARY

1. Sulfhemoglobin is formed by the action of H_2S and O_2 on hemoglobin. Prolonged action of the gas mixture destroys a part of the newly formed sulfhemoglobin, apparently by intermediary formation of H_2O_2.

2. Contrary to hemoglobin and its derivatives, sulfhemoglobin is not split into hemin and globin by dilute acids. Digestion with pepsin furnishes sulfheminproteose, i.e. a hemin, to which a part of the protein is firmly bound. In contradistinction to other green pigments (verdohemochromogens) sulfhemoglobin and sulfheminproteose lose no iron after treatment with dilute HCl.

3. Sulfhemoglobin contains 2 to 4 per cent sulfur, sulfheminproteose 7 to 10 per cent sulfur. The bulk of this sulfur is adsorbed colloidal sulfur.

4. Hydrolysis of sulfheminproteose with boiling concentrated HCl furnishes an iron-free porphyrin, which differs from protoporphyrin by an excess of 2 S and 4 O atoms. Most probably it contains two SO_2 bridges between the porphin nucleus and its side chains.

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ACETALDEHYDE IN MAMMALIAN RED BLOOD CELLS

Sirs:

Acetaldehyde has been found in whole blood of cats, dogs, rabbits, and humans, in amounts of 2 to 10 mg. per cent. At least 90 per cent of the acetaldehyde is present in the red cells, but is bound in some manner, since it is not found in the filtrates from the usual protein precipitants (trichloroacetic acid, tungstic acid, zinc hydroxide). The only reagents thus far encountered which liberate the aldehyde are alcohol and the copper sulfate-calcium hydroxide reagent of Van Slyke and Salkowski. When either of these procedures was used on whole blood or red cells, the filtrate was found to contain a volatile substance identified as acetaldehyde by means of the characteristics of its reaction with p-hydroxydiphenyl in concentrated sulfuric acid to form a violet color. A series of determinations of volatile bisulfite-binding values, calculated in terms of acetaldehyde, exactly corresponded to the colorimetric values.

Advantage was taken of the volatility of the material to concentrate it by aeration. The final filtrate had a strong odor of acetaldehyde. The identity of this substance was confirmed by the preparation of the 2,4-dinitrophenylhydrazone, m.p. 146°, and the dimedon derivative, m.p. 139°.

The amount of acetaldehyde liberated from the red cells in whole blood is about 10 times that previously reported in blood.[1] The significance of the presence of the aldehyde is wholly unknown, since it does not seem to be related to changes in blood sugar, lactate, or pyruvate. The amount in freshly shed blood remains stable for several days if stored cold. Iodoacetic acid and fluoride added as the blood is drawn have no effect on the amount of

[1] Gee, A. H., and Chaikoff, I. L., *J. Biol. Chem.*, 70, 151 (1926).

aldehyde present. Even in the presence of preservatives, the acetaldehyde value falls after prolonged storage.

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THE PRODUCTS FORMED BY THE ACTION OF OXYGEN ON COLLOIDAL SOLUTIONS OF CHOLESTEROL*

Sirs:

In a recent publication on the isolation of 7(β)-hydroxycholesterol from the serum of pregnant mares[1] we discussed the possibility, remote as it seemed to us then, that this diol may have arisen from cholesterol by air oxidation during the isolation process. The literature abounds with reports that products demonstrable, as the isolated compound is, by the Rosenheim and Lifschütz color reactions are formed from cholesterol under the influence of oxygen and heat, but the nature of these substances, generally known as "oxycholesterols," has never been established. We have now conducted experiments in which colloidal solutions of cholesterol stabilized with sodium stearate were aerated for several hours with molecular oxygen at 85°.

We were able to identify two of the products thus formed. One of them is 7(α)-hydroxycholesterol (dibenzoate m.p. 174°; $[\alpha]_D^{26} = +94.3°$ (chloroform)). This dextrorotatory isomer has been isolated from ox liver by Haslewood,[2] and more recently from hog liver by MacPhillamy.[3] Lately we have also encountered it in pregnant mare's serum.[4] Our present finding obviously renders it doubtful whether the compound really originated in the tissues mentioned. It remains to be seen whether this will also be found to be true of the levorotatory β epimer. The fact that the autoxidized material contains strongly chromogenic fractions with highly negative rotation points suspiciously to the same contingency.

The yield of chromogenic products, based on colorimetric deter-

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[2] Haslewood, G. A. D., *Biochem. J.*, **33**, 709 (1939).

[3] MacPhillamy, H. B., *J. Am. Chem. Soc.*, **62**, 3518 (1940).

[4] Wintersteiner, O., and Ritzmann, J. R., unpublished.

minations with pure 7(β)-hydroxycholesterol as standard, was consistently 25 to 30 per cent and could not be increased by further aeration. This is due to the simultaneous formation, as the main product of the reaction, of a ketone which was identified as 7-ketocholesterol (m.p. 172°; $[\alpha]_D^{23} = -104°$ (chloroform); $\epsilon = 12,500$ at 238 mμ, in alcohol). The spectroscopic assay of the crude reaction products indicated that 40 to 45 per cent of the cholesterol is transformed into the 7-ketone, and this is borne out by the good yields actually obtained. The absorption spectra also give evidence of the presence of a ketone not precipitable by digitonin and absorbing light around 280 mμ, probably 7-ketocholesterilene. Furthermore, we are investigating the possibility that small amounts of 7-dehydrocholesterol may be present in the non-ketonic fraction, which shows slight selective absorption in the same region.

These results demonstrate that position 7 in the cholesterol molecule is extremely susceptible to attack by molecular oxygen. The ease with which the autoxidation in colloidal aqueous solution takes place suggests that this reaction may be one of the pathways by which cholesterol is degraded in the organism or is converted to provitamin D_3. With this possibility in mind we hesitate to commit ourselves at present definitely as to whether the isolated 7-hydroxycholesterols should be considered as artifacts or as substances of true biological origin. In any event, the *in vitro* autoxidation of cholesterol offers promise as a convenient means for the preparation of 7-dehydrocholesterol by way of the hydroxycholesterols.

Studies on the mechanism of the reaction and on the influence of various physical and chemical factors are in progress.

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THE NATURE OF THE STIMULATION OF YEAST RESPIRATION BY CHLOROFORM-PRESERVED CYTOCHROME C EXTRACTS

Sirs:

While investigating the possibility of making cytochrome oxidase determinations of intact yeast cells, we found that a particular sample of cytochrome c^1 when added to washed culture yeast suspended in phosphate buffer medium (pH 7.2) brought about a remarkable increase in the rate of oxygen consumption. Chloroform had been used in preserving this solution of cytochrome c. Other lots of cytochrome c gave no increase in respiration when added to the washed yeast cells. These lots had not been treated with chloroform.

The addition of small amounts of chloroform-treated cytochrome c raised the rate of oxygen consumption by about 50 per cent for a short period only, after which it approached the value of the control. Washing the chloroform before it was added to the cytochrome c solution removed the oxidizable substance, indicating that a water-soluble impurity was present in the chloroform which acted as a substrate for the yeast. This impurity was the small amount of alcohol added by the manufacturer as a preservative.

The sensitivity of yeast respiration to the small amount of alcohol contained in the chloroform used to preserve cytochrome c solutions is illustrated by the following data. Shaking 0.5 ml. of chloroform with 15 ml. of buffer solution and then adding 0.4 ml. of the supernatant solution to a suspension of yeast cells respiring in Warburg vessels at 25° increased the Q_{O_2} from 9.9 to 14.4 c.mm. When 2.0 instead of 0.5 ml. of chloroform were used in the above procedure, the increase was from 9.9 to 28.5 c.mm., and the higher

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[1] All samples of cytochrome c were kindly furnished by Dr. Elmer Stotz of the Harvard Medical School and the McLean Hospital, Waverly, Massachusetts.

rate continued much longer.   The calculated amounts of alcohol added to the yeast in these experiments, the manufacturer's figure[2] (0.7 per cent) being taken for the alcohol present in the chloroform, were 0.093 and 0.373 mg.   When, in similar experiments, these amounts of pure alcohol were added to yeast cells, corresponding increases in respiration were obtained.

The stimulation of tissue respiration by the traces of alcohol in the chloroform-preserved cytochrome c extracts was also studied. Thus, the use of the chloroform-treated cytochrome c in the Stotz[3] cytochrome oxidase procedure resulted in an error of 8 per cent when rat heart and liver tissues were assayed.   The calculated amount of alcohol present was 0.2 mg. per 100 mg. of tissue (wet weight).   This amount of alcohol stimulated the oxygen consumption of rat liver slices and mash as much as 25 per cent.

Obviously, then, chloroform is not to be recommended as a preservative for solutions employed in manometric methods.

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[2] Private communication from Dr. G. W. Lewis of the Laboratory Chemical Department, Merck and Company, Inc., Rahway, New Jersey.
[3] Stotz, E., *J. Biol. Chem.*, **131**, 555 (1939).

AN ACTIVATOR OF THE HEXOKINASE SYSTEM

Sirs:

Hexokinase, the yeast factor which activates the fermentation of glucose or fructose in muscle extracts,[1] is an enzyme which is supposed to catalyze the following reaction:[2] adenyl pyrophosphate + 2 hexose → adenylic acid + 2 hexose-6-phosphate.

Hexokinase prepared from bakers' yeast by the method of Meyerhof,[1] was precipitated with saturated ammonium sulfate and the precipitate dried. The resulting powder when kept at low temperature retains its activity for at least 2 months. A purification of the hexokinase can be obtained by precipitation with ammonium sulfate between 50 and 65 per cent saturation.

In the presence of (1) hexokinase (crude as well as fractionated), (2) adenosine triphosphate, (3,a) glucose or (3,b) fructose, and (4) magnesium ions the following reaction takes place: adenosine triphosphate + hexose → adenosine diphosphate + hexose-6-phosphate (I).

Both reaction products were identified; glucose-1-phosphate is not the primary reaction product, since the enzyme necessary for its conversion to 6-phosphate is not present.

The reaction is completed within 5 minutes when 0.25 mg. of crude hexokinase protein per cc. is incubated at 30° with 2.5 mg. of glucose and adenosine triphosphate containing 60 γ of labile P, 30 γ of which are transferred to glucose. The progress of this reaction can be followed not only by the disappearance of acid-labile P but also manometrically, since one acid equivalent is liberated when one of the pyrophosphate linkages is broken with the formation of hexose-6-phosphate.

In equation (I) only one of the two labile P groups in the nucleotide is transferred to sugar, a fact which was already indi-

[1] Meyerhof, O., *Biochem. Z.*, **183**, 176 (1927).

[2] von Euler, H., and Adler, E., *Z. physiol. Chem.*, **235**, 122 (1935). Meyerhof, O., *Naturwissenschaften*, **23**, 850 (1935).

cated in some experiments of Meyerhof and Kiessling.[3] The second labile phosphate group can be transferred to hexose, provided that a heat-stable protein, present in muscle tissue, is added to the hexokinase.

The heat-stable protein in the absence of hexokinase has no activity. When adenosine diphosphate is added instead of triphosphate in the presence of hexokinase, no reaction with glucose or fructose occurs until the heat-stable protein is added; the reaction then proceeds according to the equation: adenosine diphosphate + hexose → adenylic acid + hexose-6-phosphate (II). The phosphorylated sugar, which has been isolated, contains no hexose diphosphate.

The factor necessary for reaction (II) is a protein, since it is precipitated by trichloroacetic acid, ammonium sulfate, or sodium sulfate and is destroyed by pepsin. The trichloroacetic acid precipitate of the protein when dissolved in dilute sodium hydroxide exhibits full activity, when tested immediately. The protein is inactivated in alkaline solutions but is reactivated by reduced glutathione or cysteine; the latter two substances are inactive when added without the protein. The protein retains most of its activity even after 20 minutes of boiling in 0.1 N hydrochloric acid.

The heat-stable protein is active in very low concentration; 1 γ of purified protein per cc. is able to catalyze the transfer of at least 14 γ of labile nucleotide P in 5 minutes at 30°. The protein concentration was determined by the biuret reaction. The heat-stable protein is not identical with insulin.

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[3] Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **283**, 83, table VI A (1935).

GUANIDINURIA

Sirs:

A myopathy in a young married man, 26 years old, was recently brought to our attention. During the investigation of his nitrogen metabolism, guanidine was discovered in the urine. With the isolation procedure of Sullivan, Hess, and Irreverre[1] for guanidine in urine, 52, 36, and 48 mg. (calculated from the picrate) were recovered from three 24 hour specimens, after the use of Ag_2O as an oxidizing agent. The creatine values were 1.45, 1.72, and 1.38 gm. respectively in these three specimens; for creatinine 3.57, 4.88, and 4.02 gm. were found.

Our attention was directed by Dr. Irreverre and Dr. Sullivan to studying the urine after mere filtration. *Adding a saturated aqueous solution of picric acid to the filtered urine caused a voluminous crystalline precipitate to appear immediately.* The crystals exhibited the characteristic chevrons and needles of guanidine picrate. The picrate melted at 322°, uncorrected, and at 328°, corrected. Sullivan's[2] specific test for unsubstituted guanidines was strongly positive. Nitrogen (calculated from the picrate), 29.31 per cent; theoretical 29.17. Over 571 mg. of guanidine (calculated from the picrate) have been isolated from a 24 hour urine specimen. Neither glycocyamine nor methylguanidine was detected. We cannot find any reference in the literature regarding the presence of guanidine in the uncombined form in the urine.

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[1] Sullivan, M. X., Hess, W. C., and Irreverre, F., *J. Biol. Chem.*, **114**, 633 (1936).

[2] Sullivan, M. X., *Proc. Soc. Exp. Biol. and Med.*, **33**, 106 (1935).

THE FORMATION OF PYRUVIC ACID FOLLOWING GLUCOSE INGESTION IN MAN*

Sirs:

Pyruvic acid has been shown to be a normal intermediary in the metabolism of carbohydrate *in vitro*, but this has not been definitely demonstrated *in vivo*.

We have therefore studied the blood pyruvic acid levels following glucose ingestion. Twenty-one experiments have been performed on seventeen, apparently healthy and well nourished subjects.

Glucose and pyruvic acid were determined in the blood with the subject under basal conditions. Immediately fo' wing, 1.75 gm. of glucose, per kilo of body weight, were given by mouth. This was generally administered in a 25 per cent solution. Samples were taken at 30 to 60 minute intervals, over a period of 4 to 6 hours, the subject remaining at rest in bed.

Pyruvic acid was determined by a method previously described by Bueding and Wortis.[1] The results are presented in the accompanying table.

	No of samples	Pyruvic acid per 100 cc. blood	
		Average	Range
		mg	*mg.*
Fasting	21	1 04	0 80–1.23
½ hr.	9	1 24	0 99–1 47
1 "	21	1 43	0 90–2 03
2 hrs	20	1 18	0 73–1 51
3 "	21	1 02	0 67–1.47
4 "	21	1 06	0 80–1 40
5 "	11	0 98	0 81–1 17
Peak of rise	20	1 47	1 10–2 03

The maximum rise, which averaged 0.43 mg. per 100 cc., occurred at the end of 1 hour in every case but one, in which the peak was reached in 30 minutes.   The smallest change in pyruvic acid was 0.14 mg. per 100 cc.; the greatest, 0.93 mg. per 100 cc. Following the peak, the pyruvic acid level fell, reaching the normal fasting range at, or before, the 3rd hour, in all but one case.

These observations indicate either (1) that pyruvic acid is a normal intermediary in the metabolism of glucose in man, or (2) that the ingestion of glucose by mouth stimulates the formation of pyruvic acid in some other way.

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THE RELATIVE ARGINASE ACTIVITY OF CERTAIN TUMORS AND NORMAL CONTROL TISSUES

Sirs:

The relative arginase activity of buffered extracts of the following pairs of (*a*) tumor and (*b*) normal control tissues was determined: (*a*) *transplanted* liver tumors of rats and mice, (*b*) embryonic livers and the livers of tumor-bearing and normal animals; (*a*) spontaneous mammary tumors in mice, (*b*) lactating and stilbestrol-induced hyperplastic mammary tissues; (*a*) mouse lymphomas, (*b*) lymph nodes and bone marrow. Varying ratios

Relative Arginase Activities

Tissue	$A \times 10^3$
Normal I mouse livers	72
Livers of I mice bearing transplanted liver tumor	76
" " C3H mice bearing transplanted liver tumor	69
Transplanted liver tumors in I mice	3
" " " " C3H mice	2
Normal rat livers	43
Regenerating rat livers	44
Livers of rats bearing transplanted liver tumor	36
Transplanted liver tumor in rats	4
Normal female rabbit liver	33
Liver of pregnant rabbit	28
Rabbit embryo livers (17 days old)	15
Hyperplastic mouse breast induced by stilbestrol	7
Lactating mouse breast	5
Spontaneous mammary tumor in C3H mice	22
" " " " C57xA mice	21
Normal mouse lymph nodes	2
" " bone marrow	0.4
Mouse lymphoma No. 72942	3
" " " Y103	4
" " " Y606	11

of enzyme to substrate were studied and determinations of the reaction velocities for each tissue were made.

In relatively dilute solutions there is a close proportion between the percentage hydrolysis of arginine in unit time and the cube root of the N (in mg. per cc.) of tissue extract. Reaction velocity studies were made within the concentration range in which this relation holds. The results can be described in terms of a monomolecular reaction.[1] The reaction constants were divided by the cube root of the mg. of N per cc. of each tissue extract at which the constants were determined. The quotients, A, are used as the basis for comparison of arginase activity and are given in the table.

Complete details and discussion will be given elsewhere.

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[1] Hellerman, L., and Perkins, M. E., *J. Biol. Chem.*, **112**, 175 (1935–36).

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